THE ETIOLOGY AND EPIDEMIOLOGY OF BLEEDING CANKER ON EUROPEAN BEECH

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European beech (*Fagus sylvatica*) is an important forest and landscape tree. These trees are susceptible to attack by various *Phytophthora* species. A survey of these trees in the northeastern United States shows approximately 70% of trees have symptoms of this type of infection, when patches of necrotic bark on the root flares of trunk ooze dark or rusty colored liquid. Forty percent of symptomatic bark tissue yielded a *Phytophthora* isolate. The most frequent species associated with this disease were *P. citricola* and *P. cactorum*. Multiple gene phylogenetic analysis indicated that isolates classified as P. *citricola* can be divided into two distinct clades, *P. citricola* A and B. *Phytophthora citricola* A is most frequently found associated with bleeding canker symptoms, approximately 60% of the time, followed by *P. cactorum* approximately 30% of the time. *Phytophthora citricola* B, *P. gonapodyides* and *P. cambivora* were found rarely. All of these pathogens can be found in the soil surrounding both asymptomatic and symptomatic beech, although the frequency varies. All five pathogens cause necrotic symptoms when inoculated into European beech sapling stems. In addition, *P. citricola* A and B and *P. cactorum* can cause disease on leaf and stem tissue of *Betula lenta*, *Syringa vulgaris* and *Ulmus americana*, and they are able to cause disease on leaf tissue of *Acer saccharum, Fraxinus americana, Viburnum trilobum* and *V. opulus*. In addition, *P. citricola* A and B were able to cause lesions on leaves of *Syringa reticulata* and *V. dentatum*. The

identification of the species associated with this disease and the knowledge of the role they play in the environment help as we try to develop ways to control this disease.

BIOGRAPHICAL SKETCH

Angela Nelson completed her bachelor's of science degree in biology teaching with an emphasis in botany at Brigham Young University in Provo, Utah in 2001. She then worked for two years developing science education programs for the public school and general public setting in New York State. She commenced her studies at Cornell University in 2003, where she explored course work and teaching in plant pathology, mycology and science education. Angela will continue to apply her educational philosophies to the development of science outreach and education in the fields of plant pathology and mycology.

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CHAPTER 1

INTRODUCTION

European beech (*Fagus sylvatica*) are an important component of landscapes in the northeastern United States. Recently, development of bleeding cankers and subsequent decline of trees has been blamed on infection by *Phytophthora* species (65). The research related here describes efforts to determine the species involved, and their distribution and frequency in the bark and rhizosphere of European beech.

European beech

The genus *Fagus* (Family Fagaceae), the beeches, comprises 13 species that are native to different geographic regions of the Northern Hemisphere. Eleven species are found in Asia, lending credence to the theory that beech originated there (49), although early fossil evidence can also be found in North America (76). *Fagus sylvatica*, European beech, is native to Europe (87), where it is a dominant component of man forests from southern Sweden to Italy and from Portugal to Turkey (88).

The trees have smooth gray bark and grow 30-40 meters tall (54). In forest communities, they have narrow crowns, but when grown in open sites without nearby competition for sunlight, they produce large lateral branches and full crowns (88). Life spans typically range from 200 and 400 years (102).

Depending on environmental conditions, European beech begin to produce seed when trees are between 30 and 70 years old. They can generally be found in areas where summers are humid and where the soil does not allow rapid dissipation of water (87). Their lack of drought tolerance may be due to their root systems; roots are slow growing and do not cover a large area, although they are highly branched (102).

In addition to the role of *F. sylvatica* in the structure of European forest communities, the species also has an important place in human history. *Fagus* comes from the Roman word referring to the edible nuts (88). Prehistoric spread may have been aided by the transportation of the nuts by people. Although human consumption is not typical now, the nuts continue to be an important food source for animals, and in some areas beech nut oil is still used for cooking (25). The common name, beech, comes from the Anglo-Saxon word for book, because writing tablets were made from slivers of beech wood (102). Wood has also been used for fuel and furniture making (90).

Today, European beech is more valuable as a shade and ornamental tree in landscapes and as a source of fuel and high quality construction wood in forest plantations. German forest scientists, who have long recognized the worth of the species as a cultivable crop tree, have had an interest in its propagation since the beginning of the 18th century and their efforts have led to the recognition and development of multiple cultivars including columnar and weeping forms, as well as varieties with cut leaves, purple leaves and tricolor leaves (87).

The diversity of growth habits combined with the eventual large size of open-grown specimens made this tree a popular choice for landscapes beginning in the 19th century. This trend spread to North America beginning in the Gilded Age (1875-1893) during which time increased economic prosperity led to the development of considerable wealth concentrated in the hands of an upper, elite class. These aristocrats sought to display their wealth by imitating European royalty in their home and garden designs, and European beech became a centerpiece in their gardens (89).

European beech continues to be a valuable forest tree in Europe and an important landscape tree in North America and Europe. This is due in part to the perception that it is relatively disease free. There were occasional reports of necrotic

oozing areas on the trunk and root flares caused by infection by *Phytophthora* spp., but in general it was considered to be "the tree of choice…because it is least affected by climatic hazards or pests or diseases" (87).

Belief in the resiliency of European beech has been challenged in recent years because of increased reports of European beech with decline associated bleeding cankers. Diseased and declining trees have been found in forest and landscape sites in Europe and in North America that had previously supported European beech growth. Preliminary ELISA testing and culturing efforts identified multiple *Phytophthora* species associated with this disease (65).

Phytophthora

The genus *Phytophthora* comprises a diverse group of plant pathogenic organisms, with over 80 species described (59). Genetic and morphological evidence indicate the genus is monophyletic (23), and several key features of the life cycle can be found in all members.

Phytophthora spp. have many fungal-like characteristics and for many years were placed in the kingdom Fungi. However, closer examination of morphological and biochemical features of *Phytophthora* and its relatives has led to the more recent conclusion that they are not true fungi, but rather members of the kingdom Chromista (22), order Pythiales (35). *Phytophthora* spp. produce filamentous vegetative thalli differing from fungi in that individual filaments are coenocytic and the entire thallus is diploid. Meiosis occurs in specialized gametangia, the antheridium and oogonium, just prior to fertilization (96). Sexual reproduction is homothallic in some species and heterothallic in others, but in either case a thick-walled, diploid oospore that tolerates desiccation and abrupt fluctuations in temperature is produced (113).

Asexual reproduction also occurs when specialized structures called sporangia produce swimming spores called zoospores. These zoospores differentiate in and are released from the sporangium (43). Upon release the zoospores are able to swim using their two flagella; one flagellum moves in a whiplash fashion and the other is covered with tiny hairs, or mastigonemes, and is often called the tinsel flagellum. The tinsel flagellum pulls the zoospore forward while the whiplash flagellum steers and provides additional momentum (18). These zoospores are able to sense chemical gradients and will swim in a helical pattern towards areas more conducive for growth (91). Eventually the flagella are shed, a cell wall is developed and the spore becomes a sedentary cyst (3). Cysts can persist for several weeks but sporangia and zoospores will not last more than a few days, and are highly susceptible to environmental changes in moisture and temperature (18, 113). After encystment, the zoospores will then germinate to produce mycelium, which can produce additional oospores or sporangia. Some species also produce chlamydospores, thick walled asexual spores that can also persist for longer periods of time, and can germinate under appropriate environmental conditions to produce hyphae (113).

The first species of *Phytophthora* was discovered in the context of the potato late blight epidemic in Europe in 1845. Widespread potato crop failure led to much speculation as to the cause, and although there was much controversy, the newly described microbe was identified as the pathogen by de Bary in 1861 and then in 1876 was given its current name, *Phytophthora infestans* (6). The genus name was derived from Greek and means plant destroyer (43). Additional species were then described in rapid succession, including *P. cactorum* in 1886, and *P. cambivora, P. citricola* and *P. gonapodyides* in 1927 (117). By 1930, 30 species had been described.

With the addition of new species to the genus, and with them new diseases, different models for disease development became apparent. One of the differences

was in the host range of a particular pathogen. Some species were highly specialized for a narrow range of plants, perhaps one genus or even one species, such as *P. infestans* and *P. fragariae* on *Solanum* and *Fragaria* respectively. Others had a broad host range, as typified by *P. cinnamomi* and *P. cactorum* (43, 113).

Another clear distinction was apparent in mode of inoculum dispersal. Some species, such as *P. palmivora*, *P. infestans* and *P. heveae*, produce caducous sporangia on aerial plant parts, and the sporangium itself acted as an infective propagule. Others, such as *P. megasperma* and *P. citricola,* are noncaducous and release their zoospores into the soil when soil moisture is sufficient to allow the zoospores to move freely toward potential new infection courts (7, 23). Regardless of these differences, the role of the soil environment in the development of disease has often been emphasized.

The role of inoculum in the soil is described in detail in Newhook (81). Briefly, oospores or chlamydospores reside in the soil and are able to persist there through harsh conditions such as drought and extremely cold or hot temperatures. Germination is triggered by water in the soil, possibly through rain events or, in an agricultural setting, by irrigation. The zoospores that are subsequently released are attracted to plant roots via chemical signals from the prospective hosts and will swim in the water present in the soil. The zoospores will then encyst on the surface of the root, germinate, and eventually establish infection. Infected plant tissue can also produce additional sporangia for secondary cycles of disease. In this way disease can increase exponentially if environmental and host plant conditions are conducive.

Diseases of many important agricultural crops are thought to spread through soil. For example, *P. fragariae* causes disease in strawberry and some other members of the Rosaceae by infecting the roots and causing root rot. Oospores in plant debris and in the soil can persist for 3 years or longer, and infection is more common in low

lying areas of the field where soil water content is highest (37). *Phytophthora melonis* is another pathogen that is commonly found in the soil and can infect the host plant, usually a member of the Cucurbitaceae, by invading the root tissue or other parts of the plant that are in contact with the soil (43). Heavy rainfall can trigger germination and eventual yield losses up to 80% (110). Soybean becomes infected with *P. sojae* from inoculum that persists in soil and stems of tolerant and susceptible host varieties (97), and disease progression is most rapid in areas with poor drainage or heavy rainfall (43).

Often populations of *Phytophthora* in the soil exist as a community of different species, which can lead to disease complexes, where similar symptoms of root rot result from infection by one or more species. For example, raspberry (*Rubus*) roots can be killed by multiple *Phytophthora* species: *P. cactorum, P. citricola*, *P. syringae, P. fragariae, P. megasperma, P. cambivora*, *P. cinnamomi*, *P. cryptogea, P. dreschleri*, or *P. erythroseptica* (39). Canes rooted in infested nursery soil and then planted out into fields are often the first sources of contamination (40). Further spread occurs as infested soil is moved on implements or through surface water runoff (39). Fluctuations in host resistance and environmental conditions may favor certain species over others, and waterlogging leads to higher disease incidence for all species except *P. citricola* (38).

The disease models described above are based on the assumption that the pathogen has the ability to persist in the soil. The soil provides a relatively stable environment in terms of temperature and moisture, compared to the air (41) and therefore could harbor inoculum until conditions for subsequent stages in the the disease cycle are favorable. In other words, although most research supports the idea that inoculum can persist in the soil, it is less clear if the pathogen is able to grow in the soil as a saprophyte. Gregory (53) argues that despite their ability to grow on

minimal media, *Phytophthora* species do not seem to be able to compete with other organisms and therefore are poor saprobes. This contention is supported by Lacey (71) who showed that mycelium can grow in sterilized soil, but that unsterilized soil suppressed growth. Weste (113) described the saprophytic abilities of *Phytophthora* species on a continuum, with biotrophs such as *P. infestans* and *P. sojae* as solely having a parasitic life cycle, and other species such as *P. cinnamomi* and *P. cactorum*, which have been shown to be able to grow in competition with other organisms, towards the other end of the spectrum. Some amount of competitive saprophytic ability would allow the latter species to increase in population between infection periods. Weste (113) also speculates that yet undiscovered species of *Phytophthora* might exist solely as saprophytes. The early emphasis on *Phytophthora* diseases of economically important plants does lend credence to the idea that work on the genus is biased towards species of a certain life style.

Phytophthora *spp. as pathogens of trees*

Phytophthora species are important pathogens of many tree species. Disease development is similar to that described previously, with higher disease incidence and severity associated with increased inoculum density and high soil water content. Oak decline in Europe, jarrah dieback in Australia, ink disease of chestnut in North America and Europe, and littleleaf disease of pines in North America are shown to correspond to the presence of species of *Phytophthora* in the soil (17, 57, 61, 63, 64, 82, 84, 106, 107). In addition, water-logged soil and flooding conditions create ideal conditions for zoospore movement and therefore increased disease. For example, alder decline, caused by *P. alni*, is seven times higher along riverbanks (51). Jarrah trees in Australia are more susceptible to *P. cinnamomi* in low-lying moist areas (8) and oak decline is higher in moist, clay soil (64). The incidence of *P. lateralis*

infection on Port-Orford cedar increased after heavy rains (84) and *Phytopthora inundata* is more frequently isolated from willow and olive when soil has recently been flooded (12).

The complex architecture of mature trees can lead to development of different types of symptoms. Sometimes, fine roots become infected first, and then the pathogen will grow from those minute infection courts into larger roots and then to the root collar. Ink disease of *Castanea dentata* caused by *P. cinnamomi* and *P. cambivora* (31), collar rot and basal canker of *Acer* spp. caused by *P. cactorum* (36), infection of apple by *P. megasperma* and *P. cactorum* (61), bleeding canker on dogwood caused by *P. cactorum* (99), canker of horsechestnut caused by *P. citricola* and *P. cactorum* (13) and avocado canker caused by *P. citricola* (115) are typical of this type of disease development.

Disease complexes can also occur; oak decline in Europe is a good example. Surveys of soil in oak forests revealed numerous *Phytophthora* species which have been shown to infect oak roots, including *P. quercina*, *P. citricola, P. gonapodyides* and *P. cinnamomi* (1, 2, 11, 57, 62, 64, 106). Although Camy (17) found that there was not a consistent relationship between crown status of oaks and presence of *Phytophthora* in the soil, other results have shown a significant positive correlation between decline symptoms and soil inoculum levels (11, 64).

Although the model of soil borne inoculum fits many agricultural crops and forest tree diseases well, there are some important exceptions. Most notable is *P. infestans*, the cause of potato late blight. *Phytophthora infestans* can persist as mycelium and oospores in plant debris and as oospores in the soil, but inoculum is usually wind dispersed, and epidemics are driven as copious sporangia are produced on aerial plant parts (53). This is markedly different in that disease development does not depend on zoospores swimming through free water in the soil.

Another example is *P. ramorum*, the cause of ramorum blight and sudden oak death. This pathogen was first describe by Werres *et al* (112) infecting twigs of rhododendron and viburnum in Germany and the Netherlands. The focus on this pathogen intensified when it was identified as the cause of bleeding cankers on oak in California (93). Subsequent studies have shown that this pathogen has a wide host range (33, 34, 55, 72, 103, 104). Although the pathogen can be found in the soil, it apparently does not persist there (28). Instead it appears that other infected plants act as direct sources of inoculum (50). Sporangia and chlamydospores are produced in copious amounts on the foliage of numerous other plant species, and these foliar hosts presumably act as primary sources of inoculum (92, 100). Infection can occur from splash dispersal of spores or as spores are wind blown (94). Davidson *et al* (29) showed that transmission from infected plants to leaf litter and then to a new host was common, and that the pathogen did not persist in the soil during dry periods. Also, long distance dispersal has occurred through human movement of leaf litter or through streams (29). This method of disease development suggests a new model for *Phytophthora* as a pathogen.

One unexpected consequence of recent surveys for *P. ramorum* worldwide is that numerous additional species of *Phytophthora* have been discovered. Some, such as *P. quercina*, are clearly soilborne root pathogens (64), but others, like *P. nemorosa*, have a predominantly aerial life style (56). What is puzzling about the new species is that they don't seem to have been causing enough mortality to attract attention of forest health specialists, and they may have remained unnoticed except for the increased surveys associated with increased concern about *P. ramorum*. This suggests that the breadth of the genus is poorly understood, and what knowledge we have is skewed toward those species that cause conspicuous symptoms on their hosts.

Inadvertent movement of species to new sites with potential new hosts and/or better conditions for disease contribute toward the development of epidemic diseases.

Species identification and concepts in **Phytophthora**

Identification of species in the genus *Phytophthora* can be challenging. Morphological characteristics often overlap, making identification difficult by these criteria alone (23, 43, 98). For example, *Phytophthora citricola* has been reported to include a high amount of genetic and morphological diversity. Erselius and De Vallavieille (42) used four isolates of *P. citricola* from different plant hosts in France to conduct isozyme analyses and found that all four isolates gave different patterns. Forster *et al* (47) collected 18 isolates of global distribution from plants and water, analyzed them using RFLPs, and obtained similarly high amounts of variation with no distinct groupings. Oudemans *et al* (85) examined 125 isolates from a global population from water, plants and soil using isozyme analyses, growth rate and morphology. Their results indicated that *P. citricola* comprised at least 5 distinct subgroups. Bunny (15) used 129 isolates from soil, water and plants in Australia and found three subgroups using isozymes, growth rate, morphology and aggressiveness. Balci and Halmschlager (1, 2) surveyed oak forest soils in Austria and Turkey and found that *P. citricola* isolates could be divided into three groups based on morphological characteristics, DNA analysis of the ITS regions and aggressiveness on *Quercus petraea* and *Q. cerris*. Using single strand conformational polymorphism (SSCP) on 10 isolates from plants and water in the United States, Kong *et al* (69) described four distinct subgroups within the species. Subsequent experiments found that additional isolates fell into one of these four groups. Gallegly and Hong (48) used SSCP and morphology to survey isolates from a collection. They identified three groups: *P. citricola* I, which was most common, *P. citricola* II which was typified by

an isolate from rhododendron in New York, and *P. citricola* III which was recovered from irrigation water in Oklahoma. These reports suggest that there may be several morphologically similar but genetically distinct species found within the *P. citricola* complex. However, because of the differing methods and isolates used in each of these studies it is difficult to determine correspondence among them.

More recent studies have relied at least in part on molecular techniques to aid in species identification. This has led to a shift in species concepts within the genus *Phytophthora*, from morphological groupings such as those described by Waterhouse (111) to concepts based on phylogenetic species recognition. These methods have been used for describing new species (24, 52, 67, 74), and more broadly in genus wide treatments as well (4, 23, 70). Identification of isolates using both morphological and molecular techniques is important because of the difficulty in identifying members of the genus *Phytophthora* to the species level (23). Addition of molecular data to the taxonomy tool box is especially helpful with *Phytophthora* spp. because of the lack of morphological characteristics, the plasticity of the ones that do exist, and the complication of a biological species concept by homothallism and interspecific hybridization (8, 9, 10, 12, 75).

While the ability to compare gene sequences has aided in the identification process, there are additional complications because nucleotide sequences from different regions of the genome may produce conflicting results. For example, there are continued uncertainties surrounding the relationship of *P. citricola* to *P. inflata*. Both species are similar morphologically, and, using the ITS region, Cooke *et al* (23) concluded that they were conspecific. However, analysis of additional gene regions has shown that the two species are in distinct clades (4, 70). Usually, single gene analysis cannot be presumed to give an accurate representation of evolutionary and

phylogenetic relationships; multi-gene analysis more accurately describes molecular diversity if the gene regions used are unlinked and neutral (83, 95, 101).

Phytophthora *as a cause of bleeding cankers*

Species of *Phytophthora* have been shown to cause bleeding cankers, or patches of necrotic bark that ooze fluid, on numerous tree species. There is often an assumed correspondence between these symptoms and *Phytophthora* disease (13, 63, 66) although other pathogens can cause similar symptoms (80). A large number of tree diseases with bleeding cankers have been shown to be caused by various *Phytophthora* species (Table 1.1).

Table 1.1. Tree genera with bleeding cankers attributed to infection by *Phytophthora* spp.

Host genus	Species	Source
Acer	*P. citricola, P. cactorum, P. cinnamomi, P. cambivora*	(20, 36)
Aesculus	*P. citricola, P. cactorum*	(13)
Alnus	*P. alni*	(51)
Arbutus	*P. cactorum, P. ramorum*	(99, 109)
Betula	*P. cactorum*	(19, 60)
Castanea	*P. cambivora, P. cinnamomi*	(26, 31, 108)
Citrus	*P. citrophthora, P. parasitica, P. nicotianae*	(44)
Cornus	*P. cactorum*	(27, 99)
Eleagnus	*P. cactorum*	(21)
Fagus	*P. cambivora, P. cactorum, P. citricola, P. gonapodyides, P. syringae, P. cinnamomi, P. pseudosyringae*	(65)
Lithocarpus	*P. ramorum*	(93)
Persea	*P. heveae*	(116)
Prunus	*P. syringae*	(5)
Quercus	*P. ramorum, P. nemorosa, P. citricola, P. cactorum, P. cinnamomi*	(56, 60, 77, 78, 93)
Salix	*P. cactorum*	(19)
Tilia	*P. citricola, P. cactorum*	(19)
Ulmus	*P. cactorum*	(60)

Phytophthora *as a pathogen of beech*

Phytophthora as a pathogen of European beech was first reported by Day in 1932 in England (32). He determined that *P. cambivora*, a species which had only been described 5 years previously, caused root rot on both *Fagus* and *Castanea*. Disease on the former was limited and less virulent and on the latter was more common in areas of water-logged soil, causing death when root rot was extensive. Further studies by Day indicated that incidence of the disease was linked to waterlogged and poorly draining soils (31). Inoculation of 2-4 year old *F. sylvatica* saplings with *P. cambivora* isolates from *F. sylvatica* and *Castanea sativa* resulted in development of symptoms comparable to those seen in the field. Inoculation with *P. syringae* and *P. cinnamomi* also produced symptoms, although beech was more resistant to *P. cinnamomi* (30). In addition, Day (30) showed that inoculations with *P. cambivora* conducted in April caused more seedling mortality than those conducted in July. The opposite was true for *P. cinnamomi*. To supplement seedling inoculation experiments, six mature trees were also inoculated with the same species, only *P. cambivora* produced symptoms. Based on these studies, Day recommended soil sterilization using sulphate of iron, copper and calcium sulfates or Bordeaux mixture as a means to prevent disease. Removal of infected tissue coupled with chemical treatment of the infected bark area using the compounds mentioned above could be effective if infection was not extensive. In all cases, Day recommended that proper drainage of soils should be promoted.

Day did not mention *Phytophthora* species causing problems on European beech in North America, although he did mention the occurrence of *P. cambivora* on *Castanea dentata*. Caroselli (19) described a bleeding canker of European beech in North America caused by *P. cactorum*. Caroselli's detailed description of symptoms includes observations that discoloration often extended into the sapwood and that in

beech this discoloration was often watermelon pink. However, he also noted that the discoloration did not necessarily indicate the presence of the pathogen, although it is not clear what this is based on. Caroselli's treatment recommendations differ markedly from Day's inasmuch as he strongly discouraged excision of diseased tissue and, instead, recommended application of a mixture of helione orange and malachite green, sold under the proprietary name Carosel®.

Fleischmann *et al* (46) tested European beech seedlings and saplings for response to inoculation with *P. citricola*, *P. cambivora*, *P. syringae* and *P. undulata*. They found that both *P. cambivora* and *P. citricola* caused root rot, decreased fine root mass and length, decreased gas exchange, wilt and death. *Phytophthora syringae* and *P. undulata* did not cause disease on the seedlings and saplings.

Motta and Annesi (79) described a basal bleeding canker on European beech in Italy caused by *P. pseudosyringae*, and Koch's postulates were completed with symptom production similar to that seen for *P. cambivora*. Jung *et al* (68) recovered *P. pseudosyringae* from soil, necrotic fine roots and necrotic bark of European beech. In addition, the same study concluded from soil infestation assays that *P. pseudosyringae* caused root rot on European beech, decreasing root biomass by 40%.

Jung *et al* (65) surveyed European beech in North America and Europe and found incidence of bleeding canker on sites that would not be thought to favor the disease, in that the soils were well draining and did not become waterlogged. In addition, cankers were found not only at the root collar but aerially, more than 7 m above the soil line. *Phytophthora* species in Europe were identified, in order of frequency, as *P. citricola*, *P. cambivora*, and *P. cactorum*. *Phytophthora gonapodyides*, *P. syringae* and *P. pseudosyringae* were isolated infrequently. In North America, isolates were tentatively identified as *P. inflata*, although they had numerous morphological and molecular characteristics in common with *P. citricola*, as well.

Stem inoculations with *P. citricola* and *P. inflata* were successful on both lilac (*Syringa vulgaris*) and beech. Soil infestation of beech with *P. citricola* also produced symptoms. Because diseased trees occasionally seemed to contain the invading pathogen(s) via formation of wound wood, the authors recommended that horticulturists boost host vigor by taking steps to augment overall tree health (e.g. watering during drought, draining flooded soil, aerating compacted soil, etc.).

Pathogenicity in **Phytophthora**

Many *Phytophthora* species appear to have a dominant necrotrophic phase, and therefore it may seem that they are directly causing disease due to death of plant tissue. However, especially in the polycylic disease cycle found in trees, there may be many factors coming into play. Jung *et al* (64) found that extensive deterioration of the fine roots could occur before above ground symptoms were seen. Brown and Brasier (14) found that xylem could be infected without external symptoms. Therefore, external symptoms are not a good indication of disease development.

Symptoms may also be produced by elicitins that spread through the plant ahead of colonization by the pathogen. Elicitins are small proteins produced by the pathogen that in some cases are recognized by the plant to initiate defense response (105). This was first noted by Wolf (114) who found that protein extracts from *P. nicotianae* caused necrosis on tobacco leaves. Luque *et al* (73) found that elicitins from *P. cinnamomi* caused a decline in stem growth and stomatal conductance of *Quercus suber*. Pernollet *et al* (86) found that elicitins from *P. cactorum* cause chlorosis, necrosis and wilt on tobacco leaves and Heiser *et al* (58) found that elicitins from *P. quercina* and *P. gonapodyides* caused similar symptoms of tobacco leaves, but *P. citricola* elicitins did not have a similar result. However, Fleischmann *et al* (45)

found that a single elicitin from *P. citricola* could cause necrosis, decreased gas exchange and oxidative bursts on tobacco leaves, but not on European beech leaves.

Plants with resistance to infection by *Phytophthora* may have more effective defense responses. Cahill and McComb (16) showed that phenylalanine ammonia-lyase activity, lignin concentration, and soluble phenolics increased in resistant *Eucalyptus calophylla* plants inoculated with *P. cinnamomi*, with no similar response seen in a related susceptible host species.

The precedent for one or more species of *Phytophthora* to cause disease with bleeding cankers on European beech is clear, but there are still many unanswered questions. The increasing reports of this disease in the United States necessitate action in elucidating some of the questions. In Chapter Two, I address the need for accurate information regarding the distribution of this disease in the northeastern United States, show the prevalence of the disease and the pathogens found to be associated with it, and address the identification of those pathogens through more thorough multi-gene analysis. I also explore the role of the pathogens in the soil environment. In Chapter Three, I focus on the ability of the pathogens to infect the leaf and stem tissue of multiple plant species.

REFERENCES

1. Balci Y., and Halmschlager E. 2003. Incidence of *Phytophthora* species in oak forests in Austria and their possible involvement in oak decline. Forest Pathology 33:157-174.

2. Balci Y., and Halmschlager E. 2003. *Phytophthora* species in oak ecosystems in Turkey and their association with declining oak trees. Plant Pathology 52:694-702.

3. Bartnicki-Garcia S. 1973. Cell wall genesis in natural protoplast: The zoospore of *Phytophthora palmivora*. Pages 77-91 in: Villanueva J. R., Garcia-Acha I., Gascon S., and Uruburu F., eds. Yeast, Mould, and Plant Protoplasts. Academic Press, London.

4. Blair J. E., Coffey M. D., Park S., Geiser D. M., and Kang S. 2008. A multilocus phylogeny of *Phytophthora* utilizing markers derived from complete genome sequences. Fungal Genetics and Biology 45:266-277.

5. Bostock R. M., and Doster M. A. 1984. Association of *Phytophthora syringae* with pruning wound cankers in almond. Phytopathology 74:840.

6. Bourke A. 1991. Potato blight in Europe in 1845: the scientific controversy. Pages 12-24 in: Lucas J. A., Shattock R. C., Shaw D. S., and Cooke L. R., eds. *Phytophthora*. Cambridge University Press, Cambridge.

7. Brasier C. M. 1983. Problems and prospects in *Phytophthora* research. Pages 351-364 in: Erwin D. C., Bartnicki-Garcia S., and Tsao P. H., eds. *Phytophthora*: Its Biology, Taxonomy, Ecology and Pathology. APS Press, St. Paul, Minnesota.

8. Brasier C. M., Cooke D. E. L., and Duncan J. M. 1999. Origin of a new *Phytophthora* pathogen through interspecific hybridization. Proceedings of the National Academy of Sciences 96:5868-5883.

9. Brasier C. M., Cooke D. E. L., Duncan J. M., and Hansen E. M. 2003. Multiple new phenotypic taxa from trees and riparian ecosystems in *Phytophthora gonapodyides-P. megasperma* ITS Clade 6, which tend to be high-temperature tolerant and either inbreeding or sterile. Mycological Research 107:277-290.

10. Brasier C. M., Kirk S. A., Delcan J., Cooke D. E. L., Jung T., and Man in't Veld, W. A. 2004. *Phytophthora alni* sp. nov. and its variants: designation of emerging heteroploid hybrid pathogens spreading on *Alnus* trees. Mycological Research 108:1172-1184.

11. Brasier C. M., Robredo F., and Ferraz J. F. P. 1993. Evidence for *Phytophthora cinnamomi* involvement in Iberian oak decline. Plant Pathology 42:140-145.

12. Brasier C. M., Sanchez-Hernandez E., and Kirk S. A. 2003. *Phytophthora inundata* sp. nov., a part heterothallic pathogen of trees and shrubs in wet or flooded soils. Mycological Research 107:477-484.

13. Brasier C. M., and Strouts R. G. 1976. New Record of *Phytophthora* in trees in Britain. I. *Phytophthora* root rot and bleeding canker of horse chestnut (*Aesculus hippocastanum* L.). European Journal of Forest Pathology 6:129-136.

14. Brown A. V., and Brasier C. M. 2007. Colonization of tree xylem by *Phytophthora ramorum, P. kernoviae* and other *Phytophthora* species. Plant Pathology 56:227-241.

15. Bunny F. J. 1996. The biology, ecology and taxonomy of *Phytophthora citricola* in native plant communities in Western Australia. Murdoch University, West Australia.

16. Cahill D. M., and McComb J. A. 1992. A comparison of changes in phenylalanine ammonia-lyase activity, lignin and phenolic synthesis in the roots of *Eucalyptus calophylla* (field resistant) and *E. marginata* (susceptible) when infected with *Phytophthora cinnamomi*. Physiological and Molecular Plant Pathology 40:315-332.

17. Camy C., Delatour C., and Marcais B. 2003. Relationships between soil factors, *Quercus robur* health, *Collybia fusipes* root infection and *Phytophthora* presence. Annals of Forest Science 60:419-426.

18. Carlile M. J. 1983. Motility, taxis, and tropism in *Phytophthora*. Pages 95-119 in: Erwin D. C., Bartnicki-Garcia S., and Tsao P. H., eds. *Phytophthora*: Its Biology, Taxonomy, Ecology and Pathology. APS Press, St. Paul, Minnesota.

19. Caroselli N. E. 1953. Bleeding canker disease of hardwoods. Bartlett Tree Research Laboratories Scientific Tree Topics 2:1-6.

20. Caroselli N. E., and Howard F. L. 1940. Bleeding canker of maples. Review of Applied Mycology 19:570-571.

21. Carter J. C. 1953. *Phytophthora* canker of Russian olive. Phytopathology 43:468.

22. Cavalier-Smith T. 1998. A revised six kingdom system of life. Biological Reviews 73:203-266.

23. Cooke D. E. L., Drenth A., Duncan J. M., Wagels G., and Brasier C. M. 2000. A molecular phylogeny of *Phytophthora* and related Oomycetes. Fungal Genetics and Biology 30:17-32.

24. Cooke D. E. L., Jung T., Williams N. A., Schubert R., Bahnweg G., Oßwald W., and Duncan J. M. 1999. Molecular evidence supports *Phytophthora quercina* as a distinct species. Mycological Research 103:799-804.

25. Couplan F. 1998. The encyclopedia of edible plants of North America. Keats, New Canaan, Connecticut.

26. Crandall B. S., Gravatt G. F., and Ryan M. M. 1945. Root disease of *Castanea* species and some Coniferous and Broadleaf Nursery stocks, caused by *Phytophthora cinnamomi* . Phytopathology 35:162-180.

27. Creager D. B. 1937. *Phytophthora* crown rot of dogwood. Journal of the Arnold Arboretum 18:344-348.

28. Dart N. L., Chastagner G. A., Rugarber E. F., and Riley K. L. 2007. Recovery frequency of *Phytophthora ramorum* and other *Phytophthora* spp. in the soil profile of ornamental retail nurseries. Plant Disease 91:1419-1422.

29. Davidson J. M., Wickland A. C., Patterson H. A., Falk K. R., and Rizzo D. M. 2005. Transmission of *Phytophthora ramorum* in mixed-evergreen forest in California. Phytopathology 95:587-596.

30. Day W. R. 1939. Root-rot of sweet chestnut and beech caused by species of *Phytophthora* II. Inoculation experiments and methods of control. Forestry 13:46-58.

31. Day W. R. 1938. Root-rot of sweet chestnut and beech caused by species of *Phytophthora* I. Cause and symptoms of disease: Its relation to soil conditions. Forestry 12:101-116.

32. Day W. R. 1932. The ink disease in England. Forestry 6:182.

33. Denman S., Kirk S. A., Brasier C. M., Hughs K. J. D., Griffin R., Hobdon E., and Webber J. F. 2005. Foliar infection of sweet chestnut (*Castanea sativa*) by *Phytophthora ramorum* in the UK. Plant Pathology 54:581.

34. Denman S., Kirk S. A., Brasier C. M., and Webber J. F. 2005. *In vitro* leaf inoculation studies as an indication of tree foliage susceptibility to *Phytophthora ramorum* in the UK. Plant Pathology 54:512-521.

35. Dick M. W. 2001. Straminipilous Fungi. Kluwer Academic Publishers, Dordrecht, Netherlands.

36. Drillias M. J., Kuntz J. E., and Worf G. L. 1982. Collar rot and basal canker of sugar maple. Journal of Arboriculture 8:29-33.

37. Duncan J. M., and Cowan J. B. 1980. Effect of temperature and soil moisture content on persistence of infectivity of *Phytophthora fragariae* in naturally infected field soil. Transactions of the British Mycological Society 90:336-337.

38. Duncan J. M., and Kennedy D. M. 1989. The effect of waterlogging on *Phytophthora* root rot of red raspberry. Plant Pathology 38:161-168.

39. Duncan J. M., Kennedy D. M., and Scott P. H. 1991. Relationships between non-papillate, soilborne species of *Phytophthora*: root rot of rasberry. Pages 129-147 in:

Lucas J. A., Shattock R. C., Shaw D. S., and Cooke L. R., eds. *Phytophthora*. Cambridge University Press, Cambridge.

40. Duncan J. M., Kennedy D. M., and Seemuller E. 1987. Identities and pathogenicities of *Phytophthora* spp. causing root rot of red raspberry. Plant Pathology 36:276-289.

41. Duniway J. M. 1983. Role of physical factors in the development of *Phytophthora* diseases. Pages 175-187 in: Erwin D. C., Bartnicki-Garcia S., and Tsao P. H., eds. *Phytophthora*: Its Biology, Taxonomy, Ecology, and Pathology. APS Press, St. Paul, Minnesota.

42. Erselius L. J., and De Vallavieille C. 1984. Variation in protein profiles of *Phytophthora*: comparison of six species. Transactions of the British Mycological Society 83:463-472.

43. Erwin D. C., and Ribeiro O. K. 1996. *Phytophthora* diseases worldwide. APS Press, St. Paul, Minn.

44. Fawcett H. S. 1923. Gummosis of citrus. Journal of Agricultural Research 24:191-235.

45. Fleischmann F., Koehl J., Portz R., Beltrame A. B., and Oßwald W. 2005. Physiological change of *Fagus sylvatica* seedlings infected with *Phytophthora citricola* and the contribution of its elicitin "citricolin" to pathogenesis. Plant Biology 7:650-658.

46. Fleischmann F., Schneider D., Matyssek R., and Oßwald W. F. 2002. Investigations on net CO2 assimilation, transpiration and root growth of *Fagus sylvatica* infested with four different *Phytophthora* species. Plant Biology 4:144-152.

47. Forster H., Oudemans P., and Coffey M. D. 1990. Mitochondrial and nuclear DNA diversity within six species of *Phytophthora*. Experimental Mycology 14:18-31.

48. Gallegly M. E., and Hong C. X. 2008. *Phytophthora*: Identifying species by morphology and DNA fingerprints. APS Press, St. Paul MN.

49. Gang W., and Junqing L. 1998. Distribution, regeneration and succession of *Fagus*. Journal of Forestry Research 9:290-291.

50. Garbelotto M., Davidson J. M., Ivors K., Maloney P. E., Huberli D., Koike S. T., and Rizzo D. M. 2003. Non-oak native plants are main hosts for sudden oak death pathogen in California. California Agriculture 57:18-23.

51. Gibbs J. N., Lipscombe M. A., and Peace A. J. 1999. The impact of *Phytophthora* disease on riparian populations of common alder (*Alnus glutinosa*) in southern Britain. European Journal of Forest Pathology 29:39-50.

52. Gomez-Alpizar L., Hu C., Oliva R., Forbes G., and Ristaino J. B. 2008. Phylogenetic relationships of *Phytophthora andina*, a new species from the highlands of Ecuador that is closely related to the Irish potato famine pathogen *Phytophthora infestans*. Mycologia 100:590-602.

53. Gregory P. H. 1983. Some major epidemics caused by *Phytophthora*. Pages 271-287 in: Erwin D. C., Bartnicki-Garcia S., and Tsao P. H., eds. *Phytophthora*: Its Biology, Taxonomy, Ecology, and Pathology. APS Press, St. Paul, Minnesota.

54. Grime J. P., Hodgson J. G., and Hunt R. 1988. Comparative Plant Ecology. Unwin Hyman, London.

55. Hansen E. M., Parke J. L., and Sutton W. 2005. Susceptibility of Oregon forest trees and shrubs to *Phytophthora ramorum*: a comparison of artificial inoculation and natural infection. Plant Disease 89:63-70.

56. Hansen E. M., Reeser P. W., Davidson J. M., Garbelotto M., Ivors K., Douhan L., and Rizzo D. M. 2003. *Phytophthora nemorosa*, a new species causing cankers and leaf blight of forest trees in California and Oregon, USA. Mycotaxon 89:129-138.

57. Hansen E. M., and Delatour C. 1999. *Phytophthora* species in oak forests of north-east France. Annals of Forest Science 56:539-547.

58. Heiser I., Fromm J., Giefing M., Koehl J., Jung T., and Oßwald W. 1999. Investigations on the action of *Phytophthora quercina, P. citricola* and *P. gonapodyides* toxins on tobacco plants. Plant Physiological Biochemistry 37:73-81.

59. Hong C., Richardson P. A., and Kong P. 2008. Pathogenicity to ornamental plants of some existing species and new taxa of *Phytophthora* from irrigation water. Plant Disease 92:1201-1207.

60. Howard F. L. 1942. The bleeding canker disease of hardwoods and possibilities of control. Review of Applied Mycology 21:394.

61. Jeffers S. N., Aldwinckle H. S., Burr T. J., and Arneson P. A. 1982. *Phytophthora* and *Pythium* species associated with crown rot in New York apple orchards. Phytopathology 72:533-538.

62. Jonsson U., Lundberg L., Sonesson K., and Jung T. 2003. First records of soilborne *Phytophthora* species in Swedish oak forests. Forest Pathology 33:175-179.

63. Jung T., Blaschke H., and Neumann P. 1996. Isolation, identification and pathogenicity of *Phytophthora* species from declining oak stands. European Journal of Forest Pathology 26:253-272.

64. Jung T., Blaschke H., and Oßwald W. 2000. Involvement of soilborne *Phytophthora* species in Central European oak decline and the effect of site factors on the disease. Plant Pathology 49:706-718.

65. Jung T., Hudler G. W., Griffiths H. M., Fleischmann F., and Oßwald W. 2005. Involvement of *Phytophthora* spp. in the decline of European beech in Europe and the USA. Mycologist 19:159-166.

66. Jung T., and Blaschke H. 1996. *Phytophthora* root rot in declining forest trees. Phyton 36:95-102.

67. Jung T., Hansen E. M., Winton L., Oßwald W., and Delatour C. 2002. Three new species of *Phytophthora* from European oak forests. Mycological Research 106:397-411.

68. Jung T., Nechwatal J., Cooke D. E. L., Hartmann G., Blaschke M., Oßwald W. F., Duncan J. M., and Delatour C. 2003. *Phytophthora pseudosyringae* sp. nov., a new species causing root and collar rot of deciduous tree species in Europe. Mycological Research 107:772-789.

69. Kong P., Hong C. X., Richardson P. A., and Gallegly M. E. 2003. Single-strand-conformation polymorphism of ribosomal DNA for rapid species differentiation in genus *Phytophthora* . Fungal Genetics and Biology 39:238-249.

70. Kroon, L. P. N. M., Makker F. T., van den Bosch, G. B. M., Bonants P. J. M., and Flier W. G. 2004. Phylogenetic analysis of *Phytophthora* species based on mitochondrial and nuclear DNA sequences. Fungal Genetics and Biology 41:766-782.

71. Lacey J. 1965. The infectivity of soils containing *Phytophthora infestans*. Annals of Applied Biology 56:363-380.

72. Linderman R. G., Davis E. A., and Marlow J. L. 2006. Response of selected nursery crop plants to inoculation with isolates of *Phytophthora ramorum* and other *Phytophthora* species. Hort Technology 16:216-224.

73. Luque J., Cohen M., Save R., Biel C., and Alvarez I. F. 1999. Effects of three fungal pathogens on water relations, chlorophyll fluorescence and growth of *Quercus suber* L. Annals of Forest Science 56:19-26.

74. Man in't Veld, Willem A., de Cock, Arthur W. A. M., Ilieva E., and Levesque C. A. 2002. Gene flow analysis of *Phytophthora porri* reveals a new species: *Phytophthora brassicae* sp. nov. European Journal of Plant Pathology 108:51-62.

75. Man in't Veld, Willem A., Veenbaas-Rijks W. J., Ilieva E., de Cock, Arthur W. A. M., Bonants P. J. M., and Pieters R. 1998. Natural hybrids of *Phytophthora nicotianae* and *P. cactorum* demonstrated by isozyme analysis and random amplified polymorphic DNA. Phytopathology 88:922-929.

76. Manchester S. R., and Dillhoff R. M. 2004. *Fagus* (Fagaceae) fruits, foliage and pollen from the Middle Eocene of Pacific Northwestern North America. Canadian Journal of Botany 82:1509-1517.

77. Miller P. A. 1941. *Phytophthora* bleeding canker of *Quercus agrifolia*. Phytopathology 31:863.

78. Mircetich S. M., Campbell R. N., and Matheron M. E. 1977. *Phytophthora* trunk canker of coast live oak and cork oak trees in California. Plant Disease Reporter 61:66-70.

79. Motta W., and Annesi T. 2003. A new *Phytophthora* sp. causing a basal canker on beech in Italy. Plant Disease 87:1005.

80. Nelson A. H., and Hudler G. W. 2007. A summary of North American hardwood tree diseases with bleeding canker symptoms. Arboriculture and Urban Forestry 33:122-131.

81. Newhook F. 1988. *Phytophthora* prevention and cure. Growing Today 31:31-33.

82. Oak S. W., and Tainter F. H. 1988. Risk prediction of Loblolly pine decline on littleleaf disease sites in South Carolina. Plant Disease 72:289-293.

83. O'Donnell K., Kistler H. C., Cigelnik E., and Ploetz R. C. 1998. Multiple evolutionary origins of the fungus causing Panama disease of banana: Concordant evidence from nuclear and mitochondrial gene genealogies. Proceedings of the National Academy of Sciences 95:2044-2049.

84. Oßwald W., Koehl J., Heiser I., Nechwatal J., and Fleischmann F. 2004. New insights in the genus *Phytophthora* and current diseases these pathogens cause in their ecosystem. Progress in Botany 65:436-466.

85. Oudemans P., Forster H., and Coffey M. D. 1994. Evidence for distinct isozyme subgroups within *Phytophthora citricola* and close relationships with *P. capsici* and *P. citrophthora* . Mycological Research 98:189-199.

86. Pernollet J. C., Sallantin M., Salle-Tourne M., and Huet J. C. 1993. Elicitin isoforms from seven *Phytophthora* species: comparison of their physico-chemical properties and toxicity to tobacco and other plant species. Physiological and Molecular Plant Pathology 42:53-67.

87. Peters R. 1997. Beech Forests. Kluwer Academic Publishers, Boston.

88. Poole A. L. 1987. Southern Beeches. Science Information Publication Centre, New Zealand.

89. Pregill P., and Volkman N. 1999. Landscapes in history: design and planning in the eastern and western tradition. 2nd ed. John Wiley, New York.

90. Rackham O. 1980. Ancient Woodland: its history, vegetation and uses in England. Edward Arnold, London.

91. Ribeiro O. K. 1983. Physiology of asexual sporulation and spore germination in *Phytophthora*. Pages 55-70 in: Erwin D. C., Bartnicki-Garcia S., and Tsao P. H., eds. *Phytophthora*: Its Biology, Taxonomy, Ecology and Pathology. APS Press, St. Paul, Minnesota.

92. Rizzo D. M., and Garbelotto M. 2003. Sudden oak death endangering California and Oregon forest ecosystems. Frontiers in Ecology and Environment 1:197-204.

93. Rizzo D. M., Garbelotto M., Davidson J. M., Slaughter G. W., and Koike S. T. 2002. *Phytophthora ramorum* as the cause of extensive mortality of *Quercus* spp. and *Lithocarpus densiflorus* in California. Plant Disease 86 3:205-214.

94. Rizzo D. M., Garbelotto M., and Hansen E. M. 2005. *Phytophthora ramorum*: Integrative research and management of an emerging pathogen in California and Oregon Forests. Annual Review of Phytopathology 43:309-335.

95. Rokas A., Williams B. L., King N., and Carroll S. B. 2003. Genome-scale approaches to resolving incongruence in molecular phylogenies. Nature 425:798-804.

96. Sansome E. 1965. Meiosis in diploid and polyploid sex organs of *Phytophthora* and *Achlya*. Cytolgia 30:103-117.

97. Schmitthenner A. F. 1985. Problems and progress in control of *Phytophthora* root rot of soybean. Plant Disease 69:362-368.

98. Stamps D. J., Waterhouse G. M., Newhook F. J., and Hall G. S. 1990. Revised tabular key to the species of *Phytophthora*. Mycological Papers 162:1-28.

99. Stuntz D. E., and Seliskar C. E. 1943. A stem canker of dogwood and madrone. Mycologia 35:207-221.

100. Swain S. 2002. An update on *Phytophthora ramorum*, causal agent of sudden oak death. International Oak Society Journal 13:38-45.

101. Taylor J. W., Jacobson D. J., Kroken S., Kasuga T., Geiser D. M., Hibbett D. S., and Fisher M. C. 2000. Phylogenetic species recognition and species concepts in fungi. Fungal Genetics and Biology 31:21-32.

102. Thomas P. 2000. Trees: Their Natural History. Cambridge University Press, New York.

103. Tooley P. W., Kyde K. L., and Englander L. 2004. Susceptibility of selected ericaceous ornamental host species to *Phytophthora ramorum*. Plant Disease 88:993-999.

104. Tooley P. W., and Kyde K. L. 2007. Susceptibility of some eastern forest species to *Phytophthora ramorum*. Plant Disease 91:435-438.

105. Tyler B. M. 2002. Molecular basis of recognition between *Phytophthora* pathogens and their hosts. Annual Review of Phytopathology 40:137-167.

106. Vettraino A. M., Barzanti G. P., Bianco M. C., Ragazzi A., Capretti P., Paoletti E., Luisi N., Anselmi N., and Vannini A. 2002. Occurence of *Phytophthora* species in oak stands in Italy and their association with declining oak trees. Forest Pathology 32:19-28.

107. Vettraino A. M., Morel O., Perlerou C., Robin C., Diamandis S., and Vannini A. 2005. Occurrence and distribution of *Phytophthora* species in European chestnut stands, and their association with ink disease and crown decline. European Journal of Plant Pathology 111:169-180.

108. Vettraino A. M., Natili G., Anselmi N., and Vannini A. 2001. Recovery and pathogenicity of *Phytophthora* species associated with a resurgence of ink disease in *Castanea sativa* in Italy. Plant Pathology 50:90-96.

109. Wagener W. W., and Cave M. S. 1944. *Phytophthora* canker of madrone in California. Plant Disease Reporter 28:328.

110. Wang Y., Ren Z., Zheng X., and Wang Y. 2007. Detection of *Phytophthora melonis* in samples of soil, water, and plant tissue with polymerase chain reaction. Canadian Journal of Plant Pathology 29:172-181.

111. Waterhouse G. M. 1963. Key to the species of *Phytophthora* de Bary. Mycological Papers 92:1-22.

112. Werres S., Marwitz R., Man in't Veld, W. A., De Cock, A. W. A. M., Bonants P. J. M., De Weerdt M., Themann K., Ilieva E., and Baayen R. P. 2001. *Phytophthora ramorum* sp nov., a new pathogen on rhododendron and viburnum. Mycological Research 105:1155-1165.

113. Weste G. 1983. Population dynamics and survival of *Phytophthora*. Pages 237-257 in: Erwin D. C., Bartnicki-Garcia S., and Tsao P. H., eds. *Phytophthora*: Its Biology, Taxonomy, Ecology, and Pathology. APS Press, St. Paul, Minnesota.

114. Wolf F. T. 1933. The pathology of tobacco black shank. Phytopathology 23:605-612.

115. Zentmyer G. A., Jefferson L., Hickman C. J., and Chang-Ho Y. 1974. Studies of *Phytophthora citricola*, isolated from *Persea americana*. Mycologia 66:830-845.

116. Zentmyer G. A., Klure L. J., and Pond E. C. 1978. A new canker disease of avocado caused by *Phytophthora heveae*. Plant Disease Reporter 62:918-922.

117. Zentmyer G. A. 1983. The world of *Phytophthora*. Pages 1-7 in: Erwin D. C., Bartnicki-Garcia S., and Tsao P. H., eds. *Phytophthora*: Its Biology, Taxonomy, Ecology and Pathology. APS Press, St. Paul, Minnesota.

CHAPTER 2

PREVALENCE, DISTRIBUTION, IDENTIFICATION AND INOCULATION OF *PHYTOPHTHORA* SPECIES FROM BLEEDING CANKERS OF EUROPEAN BEECH (*FAGUS SYLVATICA*) AND FROM SOIL AROUND INFECTED TREES

Abstract

While bleeding canker of European beech (*Fagus sylvatica*) has long been recognized as a problem, the cause of this disease in the northeastern United States has not been clear. We sought to survey for prevalence of this disease, to identify the pathogens involved, to prove their pathogenicity, to develop the best protocol for detecting those pathogens, and to identify if those pathogens were present in the soil surrounding European beech. Five different pathogens were found to be involved: *Phytophthora cactorum, P. gonapodyides, P. cambivora* and two distinct clades of *P. citricola* (denoted *P. citricola* A and B). *Phytophthora citricola* A and *P. cactorum* were most prevalent. All were found to cause disease when artificially inoculated into European beech sapling stems, although *P. cambivora* and *P. gonapodyides* produced significantly smaller lesions. Recovery of the pathogen from symptomatic tissue using selective media, which was significantly higher in the fall, was preferred although ELISA detection was more successful and worked regardless of season. Survey for the five pathogens in the soil surrounding European beech trees showed that all five can be found, but *P. cambivora* was most common, followed by *P. cactorum* and *P. citricola* A. These results act as the foundation for building a management strategy to protect valuable specimens of European beech.

Introduction

With any plant disease, particularly one that is newly emerging or increasing in importance, it is essential to properly identify the causal organism. This information can guide researchers to information available in the literature, dictate which questions still need to be answered, and lead to appropriate management and containment strategies. Bleeding cankers on European beech (Figure 2.1) have been reported occasionally for 75 years. The lesions have been attributed to infection by *Phytophthora cambivora* in Europe (25) and *P. cactorum* in the United States (19). More recently, additional species of *Phytophthora* have been implicated in this disease (42, 50). In addition, it is now apparent that as the bleeding cankers expand on otherwise healthy trees, leaf wilt, branch dieback, decline and death of affected trees will eventually follow (42). Anecdotal reports from the general public and tree care professionals in New York State and our own field observations suggest that the number of trees with cankers and the number of dying has increased markedly in the last decade. Because of the importance of European beech as a landscape tree in the northeastern United States, the decline of this species has caused concern.

Figure 2.1. Typical bleeding canker symptoms on the trunk of a European beech. Dotted outline area is magnified in second picture.

A first step toward development of a management program is determination of the pathogen(s) involved. In this case, identification has been impeded by difficulty in isolating the pathogen, colonization of infected plant material with secondary opportunistic pathogens, and nonspecific diagnostic tools such as ELISA. Also, the

recent discovery of *P. ramorum* and *P. kernoviae* causing bleeding cankers on European beech in England has led to heightened concerns that one of these pathogens or another potentially invasive pathogen may have spread to the United States (10). However, numerous other species of *Phytophthora* that could be involved are already established in the northeastern United States (19, 25, 42, 50). The identity of the pathogen(s) involved could have a large impact on management strategies.

Identification of the pathogen also can lead to information regarding possible sources of inoculum and the life cycle of the pathogen. For example, many species of *Phytophthora*, particularly tree pathogens, are thought to be normal components of the soil biota and to attack susceptible host plants through the roots (51). Alternatively, *P. ramorum* has been shown to spread aerially through wind and rain splash (34). Identifying the pathogen is a first step toward understanding the disease development cycle. The objectives of our research were: (i) to survey for symptomatic European beech in the northeastern United States to determine disease prevalence and distribution, (ii) to determine the best methodology for culturing or detecting the pathogen(s), (iii) to identify the pathogen(s) involved with this disease, (iv) to survey soil around European beech for the pathogen(s) and (v) to confirm the identified organisms as the pathogen(s) through completion of Koch's postulates.

Methods

Geographic distribution survey

Due to the scattered distribution of European beech in the urban and suburban landscapes, we requested assistance from landscape managers, Cooperative Extension educators and other tree care professionals in the northeast United States in our search for trees to include in this study (Table 2.1). Each location was surveyed in one of two ways. First, some sites were surveyed completely; every European beech on the site

(e.g. campus, park, residential landscape, etc.) was checked for the presence of symptoms. These complete surveys allowed for an unbiased assessment of disease frequency. In other sites, the search was limited to a smaller number of selected European beech brought to our attention by the land owners. These limited surveys allowed us to address the concerns of residents, expand the geographic area of our survey, and collect more isolates, but they were not included when calculating the frequency of diseased trees. The method of survey depended on the needs of the tree owners and limitations in accessibility. A total of 18 locations were surveyed; 11 were surveyed completely and 7 were limited to individually selected trees.

In the complete surveys, a total of 321 trees were checked for the presence of dark, necrotic and/or oozing regions on the bark (Figure 2.1) from the soil line to a height of 5 m. Data on leaf color (green- versus purple-leaf varieties) and canker aspect (cardinal and ordinal directions) were recorded to determine whether any of these factors were related to canker presence. Frequency of symptoms was then analyzed using the chi square test of independence to determine whether canker presence was independent of host leaf color or trunk aspect. In the remaining 7 locations, a total of 24 trees were selected for examination.

Table 2.1. Survey sites, trees surveyed for canker symptoms and results of those surveys.

Complete Surveys						
County	No. of trees surveyed	No. of symptomatic trees	No. of trees sampled[a]	No. of trees yielding isolates	No. of *P. cactorum* isolates	No. of *P. citricola* A isolates
Tompkins, NY[c]	61	10	7	7[b]	1	6
Albany, NY	20	10	4	2	1	1
Rensselaer, NY	11	2	2	0	-	-
Greene, NY	8	2	1	0	-	-
Erie, NY	19	6	2	1	1	0
Monroe, NY[c]	57	26	12	12	6	5
Orange, NY	7	4	4	1	0	1
Dutchess, NY	11	7	7	4	2	2
Nassau, NY[c]	99	48	20	20	4	14
Montgomery, PA	8	4	3	1	0	1
Chester, PA	20	7	6	5	1	4
Totals	321	126	68	53	16	34
Limited Surveys						
Kings, NY	n/a	n/a	1	1	0	1
Fairfield, CT	n/a	n/a	1	1	0	1
Anne Arundel, MD	n/a	n/a	2	2	0	2
Suffolk, NY	n/a	n/a	3	3	0	3
Bucks, PA	n/a	n/a	4	1	0	1
Centre, PA	n/a	n/a	3	3	0	3
Suffolk, MA[d]	n/a	n/a	10	8	1	6

[a] some trees were not sampled due to access difficulty or at the request of the owner
[b] one tree yielded two different species from two different cankers
[c] *P. gonapodyides* isolated from trees in Nassau, Monroe and Tompkins counties, NY.
[d] *P. cambivora* isolated from one tree in Suffolk county, MA.

Symptomatic trees were sampled by using a mallet and a surface disinfested chisel to cut one or more pieces of bark from the margins of cankers. Differing trunk contours did not always allow for uniformity in size of sample, but each piece was approximately 5 cm^2. Each sample was immediately placed in a plastic bag in a cooler in an air-conditioned car for transport back to the lab where it was then stored at 4°C and processed within four days.

In the lab, bark slivers were cut from the inner surface of the sample at the margin of the symptomatic tissue with a sterile razor blade, and these small (approximately 1 mm^3) pieces were placed on PARP semiselective medium (clarified V8 agar amended with pentachloronitrobenzene, ampicillin, rifampicin and pimaricin) (28). For ten days thereafter, the plates were observed for growth of hyphae from the bark into the medium. When visible hyphal growth was found, they were transferred to clarified V8 agar (28). After 3-5 days, they were examined for reproductive and/or vegetative structures indicative of *Phytophthora*. If structures similar to oogonia were observed or if hyphae remained sterile, then 5 to 7 days after transfer, a 15 mm diameter agar plug was placed in a Petri dish (60x15 mm) and flooded with sterile distilled water. One day later, the plug was examined for the presence of sporangia.

Detection method evaluation

From the initial complete surveys, a subset of 234 trees at four locations in New York (Albany, Tompkins, Monroe and Nassau Counties) were selected to determine whether frequency of *Phytophthora* detection was independent of assay method (isolation on an agar-based medium versus ELISA) and seasonality. Presence or absence of cankers were noted from each tree, and a 2.5 cm^2 bark sample was collected from the margin of each canker for isolation and ELISA tests at each of four sampling dates (July 2005, October 2005, January 2006, and May 2006). Isolations of *Phytophthora* spp. were attempted by plating bark chips from each sample on PARP

as described above. In addition, 0.1 g of finely ground dry bark from each sample was used in an ELISA test kit (Agdia, Elkhart, IN) for the detection of *Phytophthora* spp. Results of each isolation attempt and ELISA test were recorded, and differences between the two methods were analyzed using a 2X2 contingency table chi square test of independence. Contingency tables were also used to determine whether detection was independent of method and sample date for each detection method separately.

Identification of pathogens

Isolates were identified to the species level using morphological characteristics and confirmed via DNA sequencing. Features including oogonium size, oospore size, sporangium size, sporangium shape, persistence of sporangia on the sporangiophore and colony morphology on PDA were recorded. At least 30 measurements of each microscopic structure were recorded from each isolate. Characteristics were compared to descriptions in Erwin and Ribiero (28) and in Stamps *et al* (57) in order to identify each isolate to species.

Isolates were also grown in pea broth for 7-10 days (32) and DNA was extracted from the resulting mycelium following the procedure described in Smith and Stanosz (56). Resulting DNA was used as a template for polymerase chain reaction (PCR) amplification of the internal transcribed spacer (ITS) regions following the protocol described in Cooke *et al* (21). Successful amplification was confirmed by running 3 μl of the product on a 0.8% agarose gel at 80V for 30 minutes and visualizing it using ethidium bromide. The PCR product was purified using the Qiaquick PCR purification kit (Qiagen Sciences, Maryland) with the alternative, higher yield protocol and then sequenced using primers ITS4 and ITS6 (Table 2.2) at the Cornell Life Sciences Core Laboratories Center (Ithaca, NY). ITS sequences were compared to sequences published by the National Center for Biotechnology

Information (http://www.ncbi.nlm.nih.gov/) using BLAST (1). Identity based on ITS sequence data was used to confirm the morphological identifications.

Table 2.2. Primers used for PCR amplification and sequencing

Primer name	Sequence 5' → 3'
ITS	
ITS 4 (65)	TCCTCCGCTTATTGATATGC
ITS 6 (21)	GAAGGTGAAGTCGTAACAAGG
ITS 7 (21)	AGCGTTCTTCATCGATGTGC
B-tubulin	
Tubuf2 (45)	CGGTAACAACTGGGCCAAGG
Tubur1 (45)	CCTGGTACTGCTGGTACTCAG
Cytochrome c oxidase	
FM50 (48)	GTTTACTGTTGGTTTAGATG
FM75 (48)	CCTTGGCAATTAGGATTTCAAGAT
FM78 (48)	ACAAATTTCACTACATTGTCC
FM79 (48)	GGACAATGTAGTGAAATTTGT
FM80 (48)	AATATCTTTATGATTTGTTGAAA
FM83 (48)	CTCCAATAAAAAATAACCAAAAATG
FM84 (48)	TTTAATTTTTAGTGCTTTTGC
FM85 (48)	AACTTGACTAATAATACCAAA

Phylogenetic analysis of *P. citricola* isolates

A set of isolates, tentatively identified as *P. citricola* based on morphology and ITS sequence, were found to exhibit a high amount of morphological and genetic diversity. Because of this the identification of these isolates was not as clear and therefore we did additional analyses for confirmation. In addition to the ITS, two additional gene regions were amplified: β-tubulin and cytochrome c oxidase I and II with the intron (cox). β-tubulin amplifications were conducted as described by Kroon *et al* (45) with the exception that the PCR volume was increased from 25 μl to 50 μl. Cox amplifications were conducted as described by Martin and Tooley (48) with the exception that the PCR volume was increased from 50 μl to 100 μl. Presence of

amplicons was confirmed as described above. The product was cleaned as described above with the exception of the cox gene. In this case, the standard purification protocol was used and then the product was diluted with water to 100 μl. Sequencing was conducted using the two primers used for amplification, and for the cox gene, six additional primers were also used (Table 2.2). Sequencing was performed at the Cornell Biotechnology Resource Center. For all three gene regions (ITS, β-tubulin and cox), forward and reverse sequences were compared using SeqMan® and the resulting final sequences were aligned in Megalign® (DNAStar, Madison, WI) using the ClustalW algorithm and then adjusted visually.

Four alignments were created. First, the cox I region of the cox gene from three of our isolates was compared with 14 published cox I sequences of *P. citricola* and its relatives. Sequences included for comparison were from species found to be closely related by Blair *et al* (7). These sequences are published in GenBank with accession numbers listed in Table 2.3. In addition, sequence data from the type specimen of *P. citricola* (IMI 021173) were included. This analysis was conducted in order to confirm the species identification of our isolates. The other three alignments (ITS, β-tubulin, and complete cox region) were used to assess the diversity found within our isolates. Sequence data for each gene region from 35 isolates from our collection were aligned. Each sequenced gene was aligned separately.

Table 2.3. Sequences included in cox I phylogenetic analysis.

Accession Number	Species	Geographic origin	Host
AY564187	*P. inflata*	UK	*Syringa*
AY894684	*P. inflata*	Scotland	*Vaccinium vitis-idaea*
AY894685	*P. inflata*	Scotland	*Gaultheria shalon*
AY894686	*P. inflata*	Scotland	*Rhododendron ponticum*
AY894687	*P. inflata*	Scotland	*Rhododendron* sp.
AY129166	*P. capsici*	Florida	*Capsici annum*
AY564161	*P. tropicalis*	The Netherlands	*Rosa spp.*
AY564171	*P. citrophthora*	Cyprus	*Citrus limonium*
AY129173	*P. colocasiae*	China	*Colocasia esculenta*
AY564173	*P. colocasiae*	Malaysia	*Colocasia esculenta*
AY564170	*P. citricola*	South Africa	*Medicago sativa*
AY564192	*P. meadii*	India	*Hevea brasiliensis*
AY564166	*P. botryosa*	Thailand	*Hevea brasiliensis*
AY564195	*P. multivesiculata*	The Netherlands	*Cymbidium*

All four alignments were analyzed using Modeltest® (53) to check for the parameters that best fit the data. The best fit model varied by data set. These modifications were used to analyze the data sets in PAUP* (58) using maximum likelihood with 1000 replications of random sequence addition. Maximum parsimony was also conducted with the same parameters. Bootstrap analysis with 1000 replications was used to find support for tree topology. In addition, Bayesian analysis was conducted on the same data sets with the parameters from Modeltest®. The analysis was run using the MrBayes® software package for 500,000 generations and the first 50,000 generations were discarded as burn-in (54).

SSCP analysis

In order to place our analysis of diversity in the context of other work on *P. citricola*, we conducted an SSCP study as described in Kong *et al* (44). Briefly, extracted DNA of each isolate was amplified with primers ITS6 and 7 (Table 2.2). The resulting PCR product was heated at 100° C for 10 minutes, then snap frozen to allow the DNA strand to reanneal to itself instead of the opposite strand, and then run

on an 8% polyacrylamide gel. Banding patterns were visualized using silver staining (6) and compared to diagnostic banding patterns reported by Kong *et al* (44) by running reference DNA from that study in the same gel. In addition, the ITS regions, β-tubulin gene and cox gene from three isolates representing SSCP citricola groups 1, 2 and 3 were sequenced and included in the phylogenetic studies described above. DNA sequence data from the ITS region was examined for correlation between sequence differences and SSCP phenotype group.

Survey for pathogens in the soil

Five trees growing on the Cornell University campus in Ithaca, NY and 10 trees growing on the grounds of Planting Fields Arboretum, Oyster Bay (Long Island), NY were selected for a survey of *Phytophthora* populations found in the soil (Table 2.4). We had previously determined that some of the trees had cankers from which either *P. cactorum* or *P. citricola* were isolated. The remainder of the trees were either symptomatic but had not tested positive for the pathogen or were asymptomatic and apparently disease-free. One tree (site 5) was an American beech (*Fagus grandifolia*) from a forested area (Ithaca, NY); the other 14 trees were in landscape settings. Soil was sampled in a qualitative assay using the protocol described in Balci *et al* (2). Briefly, soil was collected from four points around each tree by removing the overlaying organic material and then collecting approximately 500 ml of soil to a depth of 10 cm. For each tree, soil from all four points was combined in a plastic bag for transport back to the lab. Then, each soil sample was thoroughly mixed, and a 300 ml subsample was placed into a 2 L plastic container and flooded with distilled water to at least 3 cm above the soil line. Water and soil were mixed, the soil was allowed to settle for 24-48 hours, and then all floating organic material was removed. Flooded soil was kept between 16 and 20˚C throughout the entire baiting process.

Table 2.4. Sites included in soil sample survey listed by location, and description of symptoms of the nearby host. Baiting results are listed for each sampling date and divided into the first and second baiting method attempt. Grayed boxes indicate no sample was taken on that date.

				Dec 05		Apr 06	
Site	Host	Location	Tree disease description	baiting 1	baiting 2	baiting 1	baiting 2
1	*F. sylvatica*	Ithaca	*P. citricola* A/ *P. gonapodyides*	---	---	---	---
2	*F. sylvatica*	Ithaca	Asymptomatic	*P. cactorum, P. citricola* A	---	---	---
3	*F. sylvatica*	Ithaca	*P. cactorum*	*P. cambivora*	*P. citricola* A	*P. cambivora*	*P. citricola* A
4	*F. sylvatica*	Ithaca	*P. citricola* A	---	---	*P. cactorum*	*P. citricola* A
5	*F. grandifolia*	Ithaca	Asymptomatic	---	---	---	---
6	*F. sylvatica*	Oyster Bay	symptomatic, no isolate	---	*P. cambivora, P. citricola* A		
7	*F. sylvatica*	Oyster Bay	*P. citricola* A	---	---		
8	*F. sylvatica*	Oyster Bay	*P. citricola* A	---	---		
9	*F. sylvatica*	Oyster Bay	*P. citricola* A				
10	*F. sylvatica*	Oyster Bay	*P. cactorum*				
11	*F. sylvatica*	Oyster Bay	symptomatic, no isolate	*P. citricola* B	---		
12	*F. sylvatica*	Oyster Bay	Asymptomatic				
13	*F. sylvatica*	Oyster Bay	*P. gonapodyides*				
14	*F. sylvatica*	Oyster Bay	Asymptomatic				
15	*F. sylvatica*	Oyster Bay	symptomatic, no isolate				

Table 2.4 continued.

	Jun 06		Oct 06		Mar 07	
Site	baiting 1	baiting 2	baiting 1	baiting 2	baiting 1	baiting 2
1	---	---	---	---	*P. cactorum*	*P. citricola* A
2	---	---	---	---	*P. cactorum*	*P. citricola* A
3	*P. citricola* A	*P. citrophthora*	*P. cambivora*	---	---	*P. citricola* A, *P. cambivora*
4	---	---	---	---	---	---
5	---	---			---	---
6	---	*P. cambivora*	---	---	---	---
7	*P. cactorum*	---	---	---	*P. gonapodyides*	---
8	---	---	*P. cactorum*	---	---	---
9	*P. citricola* A	*P. cambivora*	*P. cambivora*	---	*P. cambivora*	*P. citricola* A, *P. cambivora*
10	---	*P. cambivora*	---	*P. cambivora*	---	*P. cambivora*
11	*P. cactorum*, *P. gonapodyides*	---				
12	---	*P. cactorum*	---	---		
13	*P. cactorum*	---	*P. cactorum*, *P. citricola* A	---		
14	*P. cactorum*	*P. cambivora*, *P. cactorum*	*P. cactorum*	*P. cactorum*, *P. cambivora*		
15	---	---	---	*P. cambivora*		

Baits were newly emerged English oak (*Quercus robur*) leaves grown in the green house from acorns collected in Ithaca NY. Oak seedlings were periodically pruned back to encourage growth of new leaves. Leaves were removed from the oak seedlings and immediately placed on the water surface of the soil that had been flooded two days before, 5-7 leaves per soil sample. Over the next ten days, leaves were monitored for the development of discoloration (Figure 2.2). Leaves that developed discoloration were checked microscopically for the development of sporangia (Figure 2.3). When sporangia were observed, the leaf was rinsed with distilled water, blotted dry, cut into small (5 mm^2) pieces and plated onto PARPH, selective media (28). Culture dishes were monitored for growth for up to 7 days, and all cultures with characteristics of *Phytophthora* were transferred to clarified V8 agar (28) where morphological traits were used to make a tentative species identification. In addition, following methods described above, DNA from the resulting cultures was extracted and the ITS regions were sequenced to support identification.

After ten days, water was poured off of the soil and it was allowed to dry for up to 10 weeks. The soil was then reflooded and the process was repeated. Sites were sampled on five different dates: December 2005, April 2006, June 2006, October 2006, and March 2007. Some sites were not sampled on every sample date due to limitations in accessibility, but each site was sampled at least twice.

Figure 2.2. Discolored *Quercus robur* leaves floating on water above soil sample.

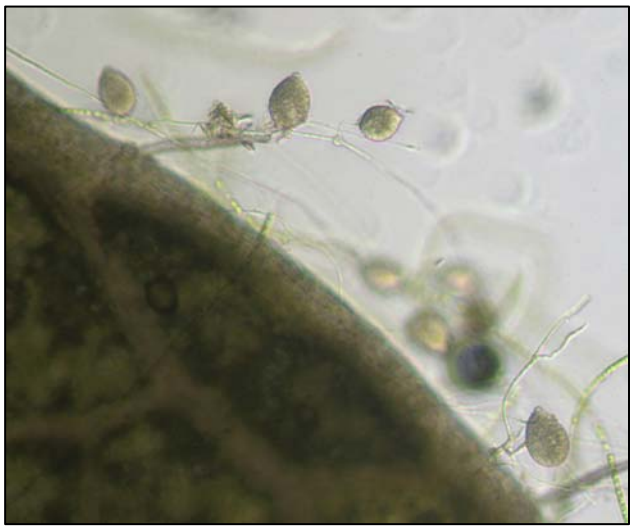


Figure 2.3. Sporulation of *Phytophthora* sp. on *Quercus robur* leaf seen at 40X.

All soil samples were baited as described above, in a first baiting attempt (baiting 1). Portions of the soil samples that were not flooded were kept at 4ºC. In September of 2007, another 300 ml from each soil sample was rebaited (baiting 2). The same protocol was used, except that after the soil had been baited and drained and dried, it was remoistened for 2 days and then reflooded. In addition, the pH of each soil sample was measured. The pH values were analyzed using a 2 sample t-test for significant differences between soil samples testing positive for *Phytophthora* and those testing negative, and for correspondence between pH level and species isolated (Minitab Statistical Software, release 15.1; Minitab Inc., State College, PA).

Completion of Koch's postulates

European beech saplings were inoculated with a representative sample of our isolates in order to complete Koch's postulates. Two isolates of *P. cactorum*¸ one isolate of *P. gonapodyides*, one isolate of *P. cambivora*, and five isolates representing two distinct clades of *P. citricola* (Table 2.5) were grown on cV8 agar for 7-10 days and then a 5 mm plug was removed from the margin of each colony. Using a 5 mm cork borer, bark was removed from the main stem of 2 year old European beech saplings and the agar plug, mycelium side in, was placed in the hole. For a control, uncolonized V8 agar plugs were used. Parafilm was wrapped around the branch to cover the wound. After two weeks the parafilm was removed and each plant was checked visually for necrotic symptom development typical of samples seen in the field. If necrosis was present, lesions were measured in the vertical direction and also for percent of stem girdle. The presence or absence of a lesions was recorded, and lesion severity was rated (1= no lesion, 2= up to 10 mm, 3=11-20 mm, 4= 20 + mm) based on a system developed by Linderman *et al* (46). This system allowed for comparison of lesion sizes between species with different leaf gross morphology. Necrotic bark on a subset of plants was removed in order to confirm that size of the

external canker corresponded with the size of the area of necrosis found within the bark. In addition, bark samples were plated on selective media (PARP) to reisolate the pathogen. Two trials were conducted, and each trial had 3 replicates of each host-pathogen combination. Severity ratings for each isolate and each species were compared using the Kruskal-Wallis test (Minitab Statistical Software, release 15.1; Minitab Inc., State College, PA).

Table 2.5. Isolates and results used for stem inoculations. Average severity ratings followed by different letters were significantly different at p=0.05 according the Kruskal-Wallis test.

Isolate	Species	% inoculations with lesion development (n=6 per isolate)	Average severity rating	St. dev. of severity rating
NYfs20	*P. cactorum*	100	2.833 ab	0.8
NYfs24	*P. cactorum*	100	3.666 a	0.8
NYfs11	*P. citricola* A	100	3.833 a	0.4
MDfs1	*P. citricola* A	100	3.833 a	0.4
NYfs18	*P. citricola* A	100	4 a	0
NYas1	*P. citricola* B	100	4 a	0
NYfs9	*P. citricola* B	100	4 a	0
Pcam	*P. cambivora*	33	1.833 b	1.3
Pgon	*P. gonapodyides*	100	2.166 b	0.4
***P. cactorum* combined**[a]		**100**	**3.25 a**	**0.9**
***P. citricola* A combined**[a]		**100**	**3.889 a**	**0.3**
***P. citricola* B combined**[a]		**100**	**4 a**	**0**

[a] Data pooled for all inoculations with all isolates of that species.

Results

Geographic distribution survey

Symptomatic trees were located at all sampling locations (Table 2.1 and Figure 2.4). Approximately 40% of European beech surveyed had bleeding cankers, with

incidence ranging from 16% in Tompkins County, NY to 64% in Dutchess County, NY. Presence of cankers was independent of leaf coloration (n=205, p=0.146) and trunk aspect (n=107, p=0.639). We did not conduct statistical analyses on all of the measured tree characteristics due to small sample sizes, but cankers were observed on trees with a variety of foliar characteristics and growth habits (e.g. fern leaf, weeping, etc.). Of the 92 symptomatic trees sampled in the complete and limited surveys, 78% yielded cultures of *Phytophthora* spp. at least once during the course of the survey (Table 2.1).

Detection method evaluation

Regardless of the season or location in which trees were sampled, detection by isolation on the semiselective medium was much less successful than by ELISA as indicated by the percentage of successful detection reported for each region over the duration of the study (Table 2.6). Chi-square analyses indicated detection was not independent of method ($p<0.001$) but was independent of location for each method (isolation $p=0.257$ and ELISA $p=0.083$). Comparison of the four different sample dates shows variation by season; isolation was most successful in the fall and least in summer (Table 2.7); however the frequency of *Phytophthora* detection by ELISA remained relatively stable across each of the four sampling dates. Chi-square analyses indicated detection was not independent of sampling date for isolation ($p<0.001$) but was independent of sampling date for ELISA ($p=0.414$).

Table 2.6. Total number of trees surveyed from July 2005 to May 2006. Percent cankered and percent of successful attempts to detect *Phytophthora*, using both isolation and ELISA methods reported over the entire survey period.

Location	No. trees surveyed	No. trees cankered (% of surveyed)	No. trees yielding cultures (% of cankered)a	No. trees ELISA positive (% of cankered) a
Albany	20	10 (50%)	1 (10%)	6 (60%)
Monroe	55	23 (42%)	8 (35%)	22 (96%)
Tompkins	61	10 (16%)	4 (40%)	7 (70%)
Nassau	98	46 (47%)	20 (43%)	36 (78%)
Total	234	89 (38%)	33 (37%)	71 (80%)

[a] sample dates and seasonality reported for each culture method in Table 2.7.
Chi-square analyses indicated detection frequency was not independent of method ($p < 0.001$), but was independent of location ($p = 0.257$ for isolation, $p = 0.083$ for ELISA).

Table 2.7. Number of trees producing cultures or positive ELISA results by method, sampling date, and location.

	Culture				ELISA			
Location	Jul 2005	Oct 2005	Jan 2006	May 2006	Jul 2005	Oct 2005	Jan 2006	May 2006
Albany	0	1	0	0	5	2	2	5
Monroe	0	8	0	1	12	16	11	9
Tompkins	1	4	3	0	6	7	6	7
Nassau	2	14	8	8	22	27	28	34
Total	3	27	11	9	45	52	9	55

Chi-square analyses indicated detection frequency was not independent of sampling date for isolation ($p < 0.001$) but was independent of sampling date for ELISA ($p = 0.414$).

Identification of pathogens

All but 4 isolates produced oogonia in a single spore culture, indicating homothallism. Antheridia were paragynous. Sporangial characteristics were more valuable for distinction of species, inasmuch as isolates with variable shaped, semipapillate sporangia were tentatively identified as *P. citricola*, and isolates with sympodially arranged, caducous, papillate sporangia as *P. cactorum* (28, 57). Most isolates fell into one of these two groups. BLAST searches of sequenced ITS regions

from all of these isolates indicated that each isolate was nearest either published *P. cactorum* or *P. citricola* sequences, confirming the identification made using sporangial morphology (1). Sequences from isolates selected to represent the geographic and species diversity were submitted to Genbank with the accession numbers listed in Table 2.8.

Table 2.8. Accession numbers and isolate collection information for ITS sequence data submitted to GenBank.

Collection #	Accession Number	County (in New York State)	Date collected	Species
NYfs24	EU044722	Tompkins	28 Sept 2004	*P. cactorum*
NYfs11	EU044726	Tompkins	5 Oct 2004	*P. citricola* A
NYfs23	EU044723	Albany	11 Sept 2004	*P. cactorum*
NYfs22	EU044725	Albany	11 Sept 2004	*P. citricola* A
NYso4	EU044724	Erie	16 Jun 2004	*P. cactorum*
NYfs25	EU044730	Monroe	31 Aug 2004	*P. cactorum*
NYfs14	EU044729	Monroe	31 Aug 2004	*P. citricola* A
NYfs26	EU044728	Dutchess	12 Oct 2004	*P. cactorum*
NYfs16	EU044727	Dutchess	12 Oct 2004	*P. citricola* A
NYfs27	EU044732	Orange	12 Oct 2004	*P. citricola* A
NYfs4	EU044721	Nassau	7 Nov 2001	*P. citricola* A
NYfs28	EU044731	Nassau	29 Oct 2005	*P. cactorum*

By combining morphological and molecular identification methods, we identified 51 isolates of *P. citricola* and 17 isolates of *P. cactorum*. These two species were found in fifteen and eight sites respectively, with seven sites yielding isolates of both species (Figure 2.4). In almost all cases, trees yielded only one species of *Phytophthora*, although one tree (Tompkins County, NY) did yield an isolate of *P. citricola* and *P. gonapodyides* (see below) from two separate cankers. In one symptomatic tree in Greene County, NY and two more trees in Rensselaer County, NY we were unable to isolate *Phytophthora*, although two trees (one in each location) were ELISA positive (Table 2.1). Some trees were sampled on more than one

occasion (see previous description of detection method evaluation) which may have increased the chances of recovering a positive culture.

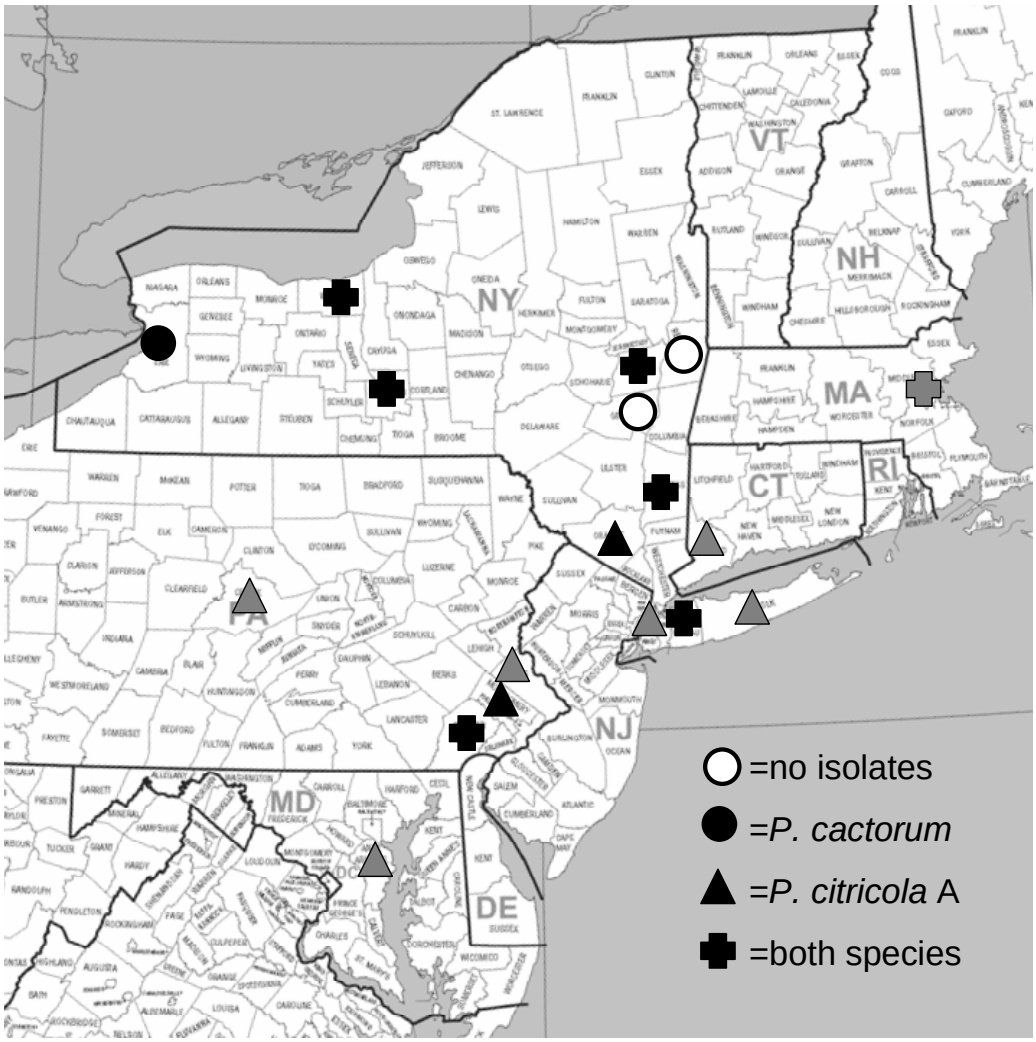


Figure 2.4. Location of surveyed counties and distribution of species recovered. White circles indicate no species was recovered, dark circles are *P. cactorum*, triangles are *P. citricola* A, and crosses are both species. Gray shapes indicate limited survey locations, black shapes are complete survey locations.

In addition to *P. cactorum* and *P. citricola*, four isolates of two other *Phytophthora* spp. were found using the same isolation and identification methods. These isolates did not produce oogonia in single spore culture, leading us to believe that they were heterothallic or sterile. Three of the isolates produced nonpapillate

sporangia and were tentatively identified as *P. gonapodyides*. The other, producing sporangia through internal proliferation, was identified as *P. cambivora*. Identification of each species was subsequently supported by DNA sequence identity to published sequences of known species.

While morphological characteristics and ITS sequence data were effective tools for identifying most isolates of *Phytophthora*, we found that isolates identified as *P. citricola* were diverse in morphological characteristics as well as in sequence data. Because of these results, we conducted further studies of the diversity found in these isolates.

Phylogenetic analysis of *P. citricola* isolates

Amplification and alignment resulted in ITS sequences 686 bp in length, β-tubulin sequences 853 bp in length and cox sequences 2112 bp in length. Alignment of just the cox I gene with published sequences results in 681 bp.

Alignment of the cox I gene region of three of our isolates with 14 published sequences indicated that our isolates were phylogenetically most similar to published sequences of *P. inflata* (Figure 2.5). *Phytophthora inflata* is a poorly characterized species assumed to be closely related to *P. citricola* (21). Sequence data from the type specimen of *P. citricola* was also closely aligned with our isolates, although another published sequence of *P. citricola* was not. Parsimony analysis of the cox I alignment found 35 most parsimonious trees (data not shown), with the strict consensus identical in topology for the clades of interest to the maximum likelihood tree shown in Figure 2.5 and the Bayesian tree.

Phylogenetic analysis of a larger group of our isolates revealed two distinct groups. All three gene regions for the 38 isolates (35 from our collection plus 3 SSCP reference isolates) had fixed polymorphisms that divided the isolates into two distinct

groups, hereafter referred to as *P. citricola* A and B. The ITS and β-tubulin genes each had three fixed differences, while cox had 23.

Maximum likelihood, maximum parsimony and Bayesian analysis of each gene separately supported the division of the isolates into two clades, *P. citricola* A and B. Because all three genes gave trees with the same topologies, the genes were concatenated and the resulting data set was analyzed as described above. The concatenated data set yielded eight most parsimonious trees and the consensus of these trees was identical in topology to the maximum likelihood tree and the Bayesian tree. This topology supported the division between the two clades, with bootstrap values of 98 and 100 for *P. citricola* A and B respectively, and posterior probability values of 100 for both (Figure 2.6).

SSCP analysis

SSCP analysis of our isolates revealed three different banding patterns, which matched the *P. citricola* groups designated by Kong *et al* (44) as Cil I, Cil II and Cil III. When SSCP data were mapped onto the phylogenetic tree produced from the three concatenated gene sequences, *P. citricola* A was a mixture of Cil I and III, while *P. citricola* B consisted solely of Cil II (Figure 2.6). Examination of the sequence data from the ITS region used in SSCP analysis revealed no sequence motif that corresponded to the SSCP Cil I and III patterns.

Figure 2.5. Phylogram based on the cox I gene. Numbers on branches indicate bootstrap value/posterior probability value. Sequences were named with species name followed by GenBank accession number. Pcitricolatype is the sequence from the *P. citricola* type specimen. Isolates from our collection are starred and labeled as follows: first two letters indicate state of origin (NY=New York, PA=Pennsylvania), second two letters indicate host plant (fs=*Fagus sylvatica*), number indicates isolate number. Shaded box indicates clade of our isolates and published *P. inflata* and *P.citricola* sequences.

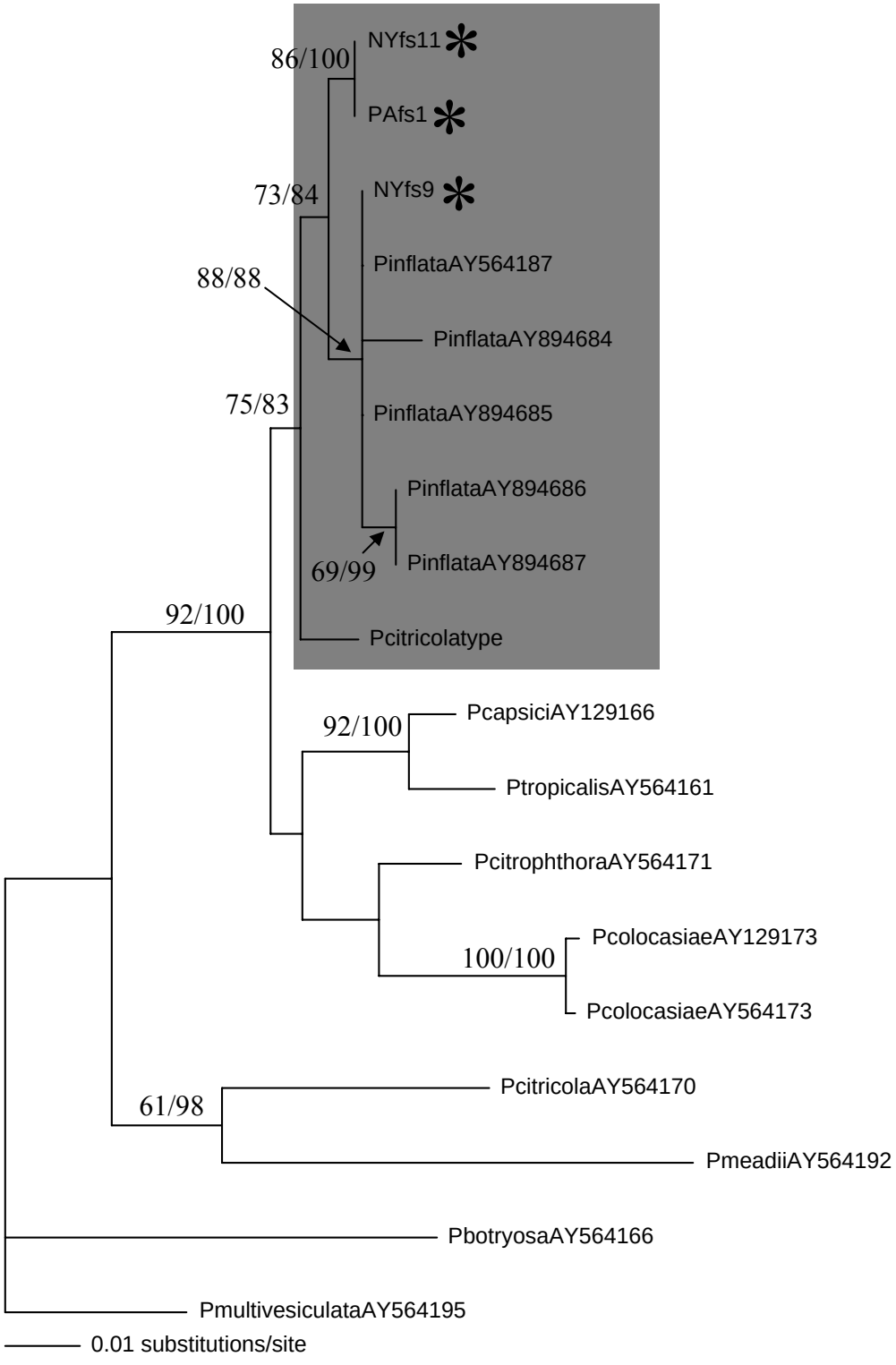
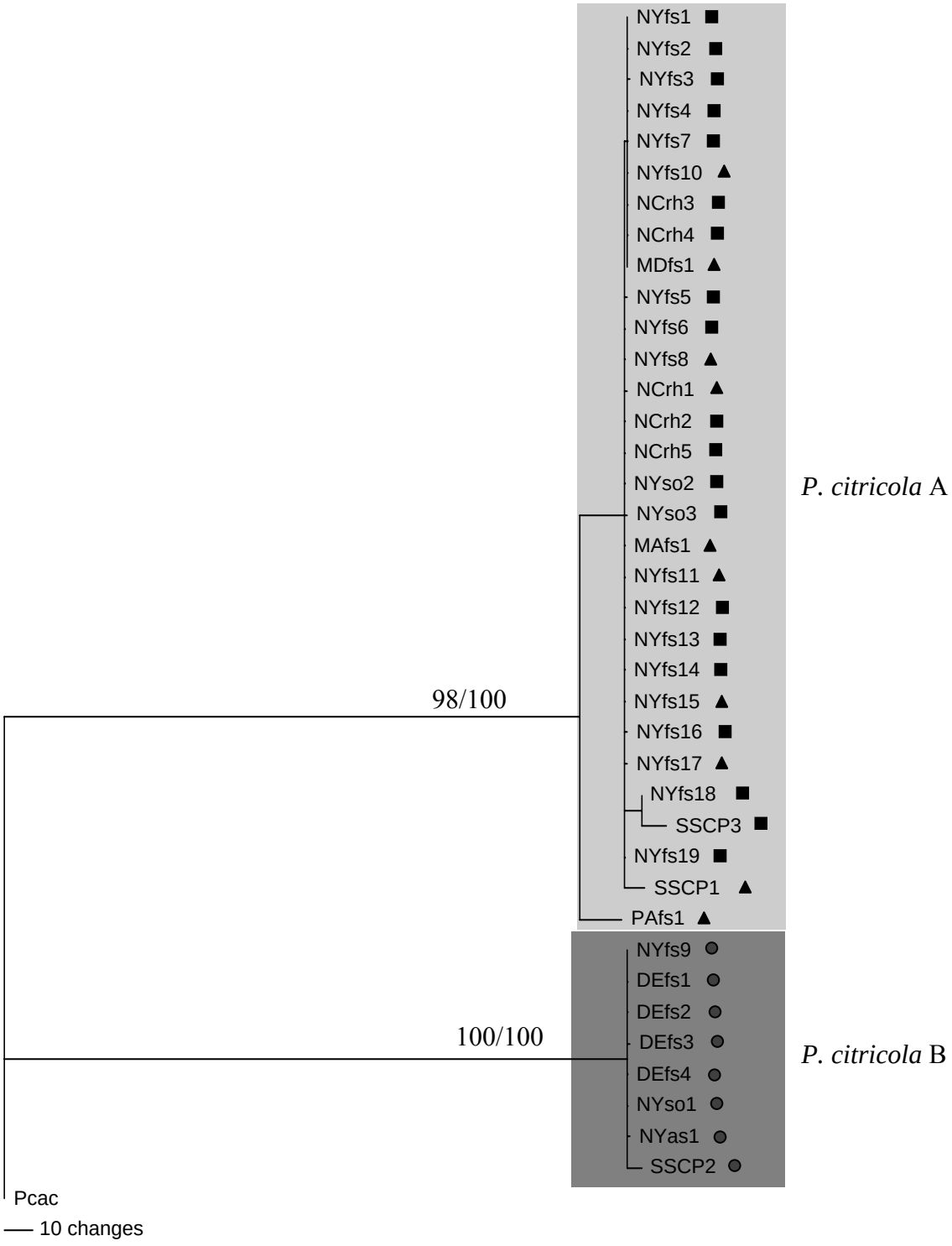

NYfs11
86/100
PAfs1
NYfs9
73/84
PinflataAY564187
88/88
PinflataAY894684
75/83
PinflataAY894685
PinflataAY894686
69/99
PinflataAY894687
92/100
Pcitricolatype
PcapsiciAY129166
92/100
PtropicalisAY564161
PcitrophthoraAY564171
PcolocasiaeAY129173
100/100
PcolocasiaeAY564173
PcitricolaAY564170
61/98
PmeadiiAY564192
PbotryosaAY564166
PmultivesiculataAY564195
0.01 substitutions/site

Figure 2.6. Maximum likelihood phylogram for three gene concatenated data set of *P. citricola* isolates. Numbers indicate bootstrap/posterior probability values. *Phytophthora citricola* B is shaded light grey and *P. citricola* A is shaded dark grey. Isolates are named using the following convention: first two letters indicate geographic region (NY=New York, MD=Maryland, DE=Germany, MA=Massachusetts, PA=Pennsylvania, NC=North Carolina), second two letters indicate where the isolate's substrate (fs=*Fagus sylvatica*, as=*Acer saccharum*, rh=*Rhododendron* sp., so=soil), number indicates isolate number. Taxa labeled SSCP1, 2 and 3 are reference isolates of Cil I, II and III from the collection of Dr. Hong. The outgroup, Pcac is an isolate of *P. cactorum* from *Fagus sylvatica* in New York. Taxa are also labeled with SSCP grouping; the triangle (▲) indicates SSCP group Cil I, the circle (●) SSCP group Cil II and the square (■) SSCP group Cil III. Our results suggest that Cil I and Cil III types are not genetically different but reflect an SSCP artifact.


NYfs1
NYfs2
NYfs3
NYfs4
NYfs7
NYfs10
NCrh3
NCrh4
MDfs1
NYfs5
NYfs6
NYfs8
NCrh1
NCrh2
NCrh5
NYso2
NYso3
MAfs1
NYfs11
NYfs12
NYfs13
NYfs14
NYfs15
NYfs16
NYfs17
NYfs18
SSCP3
NYfs19
SSCP1
PAfs1
NYfs9
DEfs1
DEfs2
DEfs3
DEfs4
NYso1
NYas1
SSCP2
98/100
100/100
P. citricola A
P. citricola B
Pcac
10 changes

Survey for pathogens in the soil

Of the 15 soil sampling sites, only one (site 5) failed to yield any *Phytophthora* isolate on any of the sample dates (Table 2.4). This site was occupied by an asymptomatic American beech in a forest setting; very different from the landscaped and managed areas that the other soil samples were taken from. The remaining 14 sites (three of which had asymptomatic trees) yielded *Phytophthora* spp. from the soil, and three yielded the same *Phytophthora* sp. as the species isolated from the cankered tree, all of which were infected with *P. citricola* A. All isolates of *P. citricola* were classified as *P. citricola* A, with the exception of one *P. citricola* B from site 11 in December 2005.

The species recovered from each site varied depending on sample date. Of all sampling sites, only 2 sites (10 and 14) yielded the same species on all sampling dates. Twelve sampling sites yielded different species across sampling dates or failed to yield any species depending on the sampling date. Of the five sample dates, June 2006 and March 2007 had the highest successful baiting rate. Success rates varied from 40-60% of the samples collected on each date. Of the 52 soil samples, 24 never yielded any *Phytophthora spp*., but 15 yielded more than one species. *Phytophthora cambivora* was the most frequently isolated species, found in 15 samples. *Phytophthora citricola* A and *P. cactorum* were found in 12 samples each, while *P. gonapodyides* was found in 2 samples and *P. citrophthora* and *P. citricola* B were found in one each.

The first baitings, conducted immediately after collection of the soil, had different results than the baiting conducted in September 2007. Although success rates (20/52 and 18/52) were similar, detection of *P. citricola* A and *P. cambivora* increased in the second baiting (from 4 to 8 and 5 to 11 respectively) while *P.*

cactorum and *P. gonapodyides* decreased in the second baiting (from 11 to 3 and 2 to 0 respectively).

The pH of the soil samples ranged from 5.21 to 7.74. No significant differences were found between the pH of soil samples testing positive for *Phytophthora* and those testing negative ($p=0.19$) or based on which species was isolated.

Completion of Koch's postulates

Inoculation of European beech saplings resulted in necrosis developing around the point of inoculation for all isolates and all pathogen species (Table 2.5). Control inoculations never developed necrosis. Excavation into the diseased bark indicated that when necrosis occurred externally, it also extended through the cambium and internal and external symptoms corresponded. Disease incidence and severity differed among isolates and pathogen species. The Kruskal-Wallis test showed that, based on the 1-4 rating scale, the severity of lesions caused by *P. cambivora* and *P. gonapodyides* was significantly lower ($p<0.01$) than the severity of lesions caused by *P. cactorum*, *P. citricola* A and *P. citricola* B. Average severity ratings for each isolate and each species that are significantly different are followed by different letters in Table 2.5. In addition, *P. cambivora* and *P. gonapodyides* did not cause girdling cankers in any instances. All sampling from necrotic lesions resulted in successful reisolation of the respective pathogen.

Discussion

The results of our survey show that bleeding cankers on European beech are common in the northeast United States, affecting approximately 40% of mature trees regardless of leaf color or growth habit. All trees that we observed with cankers were of large diameter (>40 cm), but this may be partially due to sampling artifact; large

trees were easier to find and were more likely to be brought to our attention by concerned tree owners. However, it may be that mature trees are also more susceptible to canker development or that it takes many years for cankers to develop and become visible.

ELISA testing had a higher success rate than isolation for detecting the presence of *Phytophthora* in cankers throughout the year. However, pathogen isolation from symptomatic tissue has the advantage of yielding a culture that can be used for subsequent analyses including species identification and pathogenicity trials. Isolation was most successful in October. Previous studies have shown that *P. citricola* and *P. cactorum* do have seasonal fluctuations in ability to colonize apple and walnut, with growth rates highest in late spring and summer (31, 37, 49). El Hamalawi and Menge (26) found growth of *P. citricola* on avocado had two peaks, one in May-June and another in November-December. This suggests that these pathogens may be more actively growing on European beech during certain times of year, making them easier to obtain from symptomatic tissue in the fall.

Identification of isolates using both morphological and molecular techniques is important because of the difficulty in identifying members of the genus *Phytophthora* to the species level (21). Morphological characteristics often overlap, making identification by these criteria alone difficult (21, 28, 57), while nucleotide sequences from different regions of the genome may produce conflicting results when used for species identification (45). Morphological characteristics, particularly sporangial morphology, were helpful in identifying isolates of *P. cactorum*, *P. gonapodyides*, and *P. cambivora* in this study, but the diversity seen among isolates classified as *P. citricola* warranted further investigation. *Phytophthora citricola* has been reported to include a high amount of diversity (4, 5, 16, 27, 29, 30, 44, 52). In addition, analysis of the ITS region has suggested that *P. citricola* may be conspecific with *P. inflata*

(21), although other gene regions provide evidence that they are distinct (7, 45). Morphological differences such as the inflated antheridium and the coiled antheridial stalk were found occasionally in our study, but too inconsistently to be diagnostic of these species as defined in the original description of *P. inflata* (20). In addition, the ability to produce sporangia on agar, a trait found in *P. inflata* but not *P. citricola* (28), was found in all cultures. Gallegly and Hong (30) suggest further morphological traits to distinguish between distinct groupings of *P. citricola*, but these characteristics were not observed in our isolates.

In order to clarify our identification of these isolates, we compared cox I sequences with published sequences of *P. citricola*, *P. inflata* and closely related species. We selected the cox I gene because, for the species in question, it was the gene with the best representation in GenBank after the ITS region. The ITS region did not provide sufficient resolution (data not shown). The cox I analysis indicated that our sequences were most closely related to published *P. inflata* sequences. However, only one cox I sequence of *P. citricola* was available in GenBank. Other studies have demonstrated high intraspecific diversity in *P. citricola* (4, 5, 16, 27, 29, 30, 44, 52). Inclusion of sequence data from the extype culture of *P. citricola* shows that it does align closely with our isolates. While the published sequence is from an isolate found on alfalfa in South Africa, the type specimen is from orange in Taiwan (Table 2.3). Therefore, the outcome of this analysis could be highly dependent on the origin and correct identification of the species of the sequences used. Given the morphological data and the sequence data, the isolates in our collection do represent members of the *P. citricola* species complex. Further study is needed to define the concepts of *P. citricola* and *P. inflata*.

The multiple gene analyses we conducted indicate that the isolates initially classified as *P. citricola* are better described as two well-supported clades that are

separated by at total 29 fixed differences among all three gene regions. The two groups could be considered two distinct phylogenetic species. The concordance of multiple gene trees indicates that polymorphisms have become fixed across the genome between the two clades, suggesting reproductive isolation and supporting species separation (36, 60). In addition, the *Phytophthora* ITS region accumulates mutations at a rate that approximates that of speciation, and the number of polymorphisms seen in this region is comparable to differences seen between well established *Phytophthora* species (15, 21, 22).

Additional evidence for species distinction such as morphological differences and reproductive barriers are lacking in the isolates used in this study. While morphological variation occurred within and among isolates, no phylogenetic correspondence with morphological characteristics was found. It is difficult to apply a biological species concept because *P. citricola* is homothallic and readily produces sexual reproductive structures in single spore cultures. In addition, some other species of *Phytophthora* have the ability to hybridize, thus complicating use of a mating test for species discrimination (8, 9, 11, 13, 47). However, because both *P. citricola* A and B were found in the same location and on the same host, there appears to be an opportunity for sexual reproduction between these two groups. The lack of hybrids in our sample and the fixed polymorphisms between *P. citricola* A and B suggest that something is acting or did act as a reproductive barrier.

We conducted SSCP analysis in order to determine if our conclusions corresponded with previous studies (44). *Phytophthora citricola* B corresponded with SSCP group Cil II, but *P. citricola* A included both SSCP groups Cil I and Cil III. However, the sequence of the gene region used in the SSCP analysis includes no corresponding sequence differences between SSCP group Cil I and Cil III. Therefore, these results may arise from an unknown artifact. We can offer no explanation why

different SSCP phenotypes would be produced without a corresponding sequence motif, even though SSCP is supposed to reflect differences in sequence. Although SSCPs may be useful for identification, this finding suggests they are subject to artifacts and raises questions as to their utility for inferring species identity.

While all of the isolates from Germany fall into the *P. citricola* B clade, this may reflect the small sample size (n=4) rather than geographic structure. This is supported by the fact that Balci and Halmschlager found three types of *P. citricola* in Turkey and two types in Austria (4, 5). Collectively, these types corresponded to the SSCP groups Cil I, Cil II and Cil III (Balci, personal communication). More intensive sampling of *P. citricola* in Eurasia and North America is needed to address geographic range.

In contrast to results from European surveys, where three species of *Phytophthora* (*P. cactorum*, *P cambivora*, and *P. citricola*) are usually associated with bleeding cankers of European beech (42), we consistently found only two species were predominant in the northeastern United States; *P. cactorum* and *P. citricola* A. The presence of *P. cactorum* on approximately one third of the surveyed trees in the present study confirms the observation initially made by Caroselli in 1953 (19). It also adds to previous research by this lab (42), which indicated that only *P. citricola* (reported then as *P. inflata*) was associated with the current outbreak of the disease. *Phytophthora cambivora* was only isolated once during our survey and was not widely encountered as might be suggested by reports from Europe (25, 42).

Results of our soil surveys present a different picture of *Phytophthora* species in the European beech rhizosphere. In soil, the most frequently isolated species was *P. cambivora*. It was found in 15 out of 52 soil samples. However, it was only found at 6 of the 15 sites, indicating it is perhaps not as common but is easier to detect through baiting with English oak leaves. Also, it is unclear why this pathogen, which has been

documented to cause bleeding cankers on European beech (25, 42), is not an important factor in this disease in the northeastern United States. However, none of the sites sampled contained trees that had been found to be infected with *P. cambivora*. *Phytophthora citricola* B was only recovered from soil at one site on one sample date, but trees infected with *P. citricola* B were not represented in the soil survey due to the small number of trees with this type of infection and difficulty in accessing them.

Successful isolation of both *P. citricola* A and *P. cactorum* from twelve soil samples each suggests that baiting with *Q. robur* leaves is capable of detecting these two European beech pathogens in soil samples. *Phytophthora cactorum* was isolated from 9 out of 15 sites, and *P. citricola* A was isolated from 8 out of 15 sites. The selective media used in this study, PARPH, contains hymexazol, which has been reported to inhibit growth of *P. cactorum* (33, 38). However, our results indicate that it is able to grow on this media, in accordance with Tay *et al* (59) and Jung *et al* (41). Differential sensitivity to hymexazol among isolates of *P. cactorum* may have led us to underestimate the prevalence of this species.

While there is not a full correlation between soil pathogen populations and diseased trees, other studies have found that significant associations exist between soil populations of *Phytophthora* and tree health (2, 4, 5). Also, the changes observed in results between sample dates correspond with previous studies which found seasonal fluctuations in soil populations (2, 18, 55, 63, 64). Because this soil assay did not measure the quantity of inoculum in the soil, it is not possible to determine if fluctuations in population size were also occurring.

Of particular interest are the three asymptomatic trees that yielded *Phytophthora* from the soil. This suggests that symptom development may not occur as the immediate result of deposition of pathogenic inoculum in the vicinity of a susceptible tree. Trees may exhibit symptoms of infection only during times of stress

or after they have been infected for an extended period of time. Alternatively, trees may be able to resist infection in some cases. Additional surveys over a longer period of time and including healthy and diseased trees are needed to confirm the timeline of disease development.

Almost half of the soil samples (24 out of 52) did not yield any species of *Phytophthora.* This may be due to the detection method; baiting may not be very efficient at detecting *Phytophthora* at low levels, particularly if there are many other species in the soil which may out compete *Phytophthora* in colonizing the oak leaves used as bait. The type of bait we selected could also have affected the baiting results in terms of number of positive soil samples and species recovered. In addition, the volume of soil sampled may not have been sufficient to include all *Phytophthora* species associated with the rhizosphere; however, the pooling of four samples combined with the thorough mixing of the samples from each tree may have helped to limit error caused by this. Baiting is the most common method for surveying soils for *Phytophthora* because of the method's ability to eliminate faster growing saprobic fungi found in soil (2, 4, 5, 12, 17, 18, 35, 39, 40, 41, 62, 63) and this specific protocol has been used by Balci *et al* (2) with success rates similar to ours. Baiting also has advantages because the resulting culture can be identified using morphological traits and multiple gene sequences, and then used in pathogenicity assays.

Completion of Koch's postulates indicates *P. citricola* A, *P. citricola* B, *P. cactorum*, *P. gonapodyides*, and *P. cambivora* can cause symptoms on European beech stems. While these results seem to indicate that these pathogens are responsible for bleeding cankers on European beech, there are some important distinctions. First, this work was done on saplings. Due to the high value placed on mature specimens of this tree, we were not able to inoculate any such trees. However, saplings are frequently used in inoculation tests involving tree diseases (3, 5, 40, 42, 43, 61). In

addition, while we did see necrosis develop, no bleeding developed except in very few cases. This may be due to the different age of the host, and the effect this has on symptom development; a sapling is girdled much more quickly than a mature tree. Bleeding may develop when pathogen colonization has the opportunity to develop to a certain extent with the tree still being alive and maintaining certain physiological processes.

Isolates of *P. gonapodyides* and *P. cambivora* were significantly less aggressive as measured by canker size. This is surprising for *P. cambivora* in particular, because it has a strong historical record of causing bleeding cankers on European beech (23, 24, 25, 42). The isolates used in this study were from soil, and therefore they might not be as aggressive as isolates from bleeding cankers. Koch's postulates have been completed previously for *Phytophthora gonapodyides* and *P. cambivora* (23, 43) using stem inoculations which yielded typical necrotic symptoms.

To our knowledge, this is the first record of Koch's postulates being completed using *P. cactorum* in stem inoculations on European beech. *Phytophthora citricola* has been shown to cause lesions on European beech stems in the past (43) but these results have an added value because two distinct clades of *P. citricola* were tested and confirmed to cause disease. Given the broad species concept of *P. citricola*, it was important to know that the particular clades found in this study were pathogenic.

In addition to *P. cactorum*, *P. cambivora*, *P. gonapodyides* and *P. citricola*, European forest surveys have reported *P. syringae*, *P. pseudosyringae*, *P. ramorum* and *P. kernoviae* causing bleeding cankers on European beech (10, 14, 42, 50). In view of the perceived threat of the latter two species to North American forests, understanding the current causes and characteristics of this disease is essential. Continued surveying for symptoms with identification to the species level is an need.

The sampling and detection methodology described in this study will be important in these efforts.

This study has shown the pathogens involved in bleeding cankers of European beech in the northeastern United States. The accurate identification of these pathogens is valuable in creating an appropriate response and treatment strategy. Understanding the possible reservoirs and sources of this inoculum in the soil also helps to build a disease development model as we seek to disrupt the cycle of this disease.

REFERENCES

1. Altschul S. F., Gish W., Miller W., Myers E. W., and Lipman D. J. 1990. Basic local alignment search tool. Journal of Molecular Biology 215:403-410.

2. Balci Y., Balci S., Eggers J., and MacDonald W. L. 2007. *Phytophthora* spp. associated with forest soils in Eastern and North-Central U.S. oak ecosystems. Plant Disease 91:705-710.

3. Balci Y., Balci S., MacDonald W. L., and Gottschalk K. W. 2008. Relative susceptibility of oaks to seven species of *Phytophthora* isolated from oak forest soils. Forest Pathology 38:394-409.

4. Balci Y., and Halmschlager E. 2003. Incidence of *Phytophthora* species in oak forests in Austria and their possible involvement in oak decline. Forest Pathology 33:157-174.

5. Balci Y., and Halmschlager E. 2003. *Phytophthora* species in oak ecosystems in Turkey and their association with declining oak trees. Plant Pathology 52:694-702.

6. Beidler J. L., Hilliard P. R., and Rill R. L. 1982. Ultrasensitive staining of nucleic acis with silver. Analytical Biochemistry 126:374-389.

7. Blair J. E., Coffey M. D., Park S., Geiser D. M., and Kang S. 2008. A multilocus phylogeny of *Phytophthora* utilizing markers derived from complete genome sequences. Fungal Genetics and Biology 45:266-277.

8. Brasier C. M., Cooke D. E. L., and Duncan J. M. 1999. Origin of a new *Phytophthora* pathogen through interspecific hybridization. Proceedings of the National Academy of Sciences 96:5868-5883.

9. Brasier C. M., Cooke D. E. L., Duncan J. M., and Hansen E. M. 2003. Multiple new phenotypic taxa from trees and riparian ecosystems in *Phytophthora gonapodyides-P. megasperma* ITS Clade 6, which tend to be high-temperature tolerant and either inbreeding or sterile. Mycological Research 107:277-290.

10. Brasier C. M., Denman S., Brown A., and Webber J. 2004. Sudden oak death (*Phytophthora ramorum*) discovered on trees in Europe. Mycological Research 108:1108-1110.

11. Brasier C. M., Kirk S. A., Delcan J., Cooke D. E. L., Jung T., and Man in't Veld, W. A. 2004. *Phytophthora alni* sp. nov. and its variants: designation of emerging heteroploid hybrid pathogens spreading on *Alnus* trees. Mycological Research 108:1172-1184.

12. Brasier C. M., Robredo F., and Ferraz J. F. P. 1993. Evidence for *Phytophthora cinnamomi* involvement in Iberian oak decline. Plant Pathology 42:140-145.

13. Brasier C. M., Sanchez-Hernandez E., and Kirk S. A. 2003. *Phytophthora inundata* sp. nov., a part heterothallic pathogen of trees and shrubs in wet or flooded soils. Mycological Research 107:477-484.

14. Brasier C. M., Beales P. A., Kirk S. A., Denman S., and Rose J. 2005. *Phytophthora kernoviae* sp. nov., an invasive pathogen causing bleeding stem lesions on forest trees and foliar necrosis of ornamentals in the UK. Mycological Research 109:853-859.

15. Bruns T. D., White T. J., and Taylor J. W. 1991. Fungal Molecular Systematics. Annual Review of Ecology and Systematics 22:525-564.

16. Bunny F. J. 1996. The biology, ecology and taxonomy of *Phytophthora citricola* in native plant communities in Western Australia. Murdoch University, West Australia.

17. Campbell W. A., and Gallegly M. E. 1965. *Phytopthora heveae* from Eastern Tennessee and western North Carolina. Plant Disease Reporter 49:233-234.

18. Camy C., Delatour C., and Marcais B. 2003. Relationships between soil factors, *Quercus robur* health, *Collybia fusipes* root infection and *Phytophthora* presence. Annals of Forest Science 60:419-426.

19. Caroselli N. E. 1953. Bleeding canker disease of hardwoods. Bartlett Tree Research Laboratories Scientific Tree Topics 2:1-6.

20. Caroselli N. E., and Tucker C. M. 1949. Pit canker of elm. Phytopathology 39:481-488.

21. Cooke D. E. L., Drenth A., Duncan J. M., Wagels G., and Brasier C. M. 2000. A molecular phylogeny of *Phytophthora* and related Oomycetes. Fungal Genetics and Biology 30:17-32.

22. Cooke D. E. L., and Duncan J. M. 1997. Phylogenetic analysis of *Phytophthora* species based on ITS1 and ITS2 sequences of the ribosomal RNA gene repeat. Mycological Research 101:667-677.

23. Day W. R. 1939. Root-rot of sweet chestnut and beech caused by species of *Phytophthora* II. Inoculation experiments and methods of control. Forestry 13:46-58.

24. Day W. R. 1938. Root-rot of sweet chestnut and beech caused by species of *Phytophthora* I. Cause and symptoms of disease: Its relation to soil conditions. Forestry 12:101-116.

25. Day W. R. 1932. The ink disease in England. Forestry 6:182.

26. El Hamalawi Z. A., and Menge J. A. 1995. Seasonal fluctuations in the extent of colonization of avocado plants by the stem canker pathogen *Phytophthora citricola*. Journal of the American Society for Horicultural Science 120:157-162.

27. Erselius L. J., and De Vallavieille C. 1984. Variation in protein profiles of *Phytophthora*: comparison of six species. Transactions of the British Mycological Society 83:463-472.

28. Erwin D. C., and Ribeiro O. K. 1996. *Phytophthora* diseases worldwide. APS Press, St. Paul, Minn.

29. Forster H., Coffey M. D., Ellwood H., and Sogin M. L. 1990. Sequence analysis of the small subunit ribosomal RNAs of three zoosporic fungi and implications of fungal evolution. Mycologia 82:306-312.

30. Gallegly M. E., and Hong C. X. 2008. *Phytophthora*: Identifying species by morphology and DNA fingerprints. APS Press, St. Paul MN.

31. Gates J. E., and Millikan D. F. 1972. Seasonal fluctuations in susceptibility of the inner bark tissues of apple to colonization by the collar rot fungus, *Phytophthora cactorum*. Phytoprotection 53:76-81.

32. Goodwin S. B., Drenth A., and Fry W. E. 1992. Cloning and genetic analyses of two highly polymorphic, moderately repetitive nuclear DNAs from *Phytophthora infestans*. Current Genetics 22:107-115.

33. Hansen E. M., Hamm P. B., Julis A. J., and Roth L. F. 1979. Isolation, incidence and management of *Phytophthora* in forest tree nurseries in the Pacific northwest. Plant Disease Reporter 63:607-611.

34. Hansen E. M. 2008. Alien forest pathogens: *Phytophthora* species are changing world forests. Boreal Environment Research 13:33-41.

35. Hansen E. M., and Delatour C. 1999. *Phytophthora* species in oak forests of north-east France. Annals of Forest Science 56:539-547.

36. Harrington T. C., and Rizzo D. M. 1999. Defining species in the Fungi. Pages 43-71 in: Worrall J. J., ed. Structure and Dynamics of Fungal Populations. Kluwer Academic Publishers, Netherlands.

37. Jeffers S. N., and Aldwinckle H. S. 1986. Seasonal variation in extent of colonization of two apple rootstocks by five species of *Phytophthora*. Plant Disease 70:941-945.

38. Jeffers S. N., and Martin S. B. 1986. Comparison of two media selective for *Phytophthora* and *Pythium* species. Plant Disease 70:1038-1043.

39. Jonsson U., Lundberg L., Sonesson K., and Jung T. 2003. First records of soilborne *Phytophthora* species in Swedish oak forests. Forest Pathology 33:175-179.

40. Jung T., Blaschke H., and Neumann P. 1996. Isolation, identification and pathogenicity of *Phytophthora* species from declining oak stands. European Journal of Forest Pathology 26:253-272.

41. Jung T., Blaschke H., and Oßwald W. 2000. Involvement of soilborne *Phytophthora* species in Central European oak decline and the effect of site factors on the disease. Plant Pathology 49:706-718.

42. Jung T., Hudler G. W., Griffiths H. M., Fleischmann F., and Oßwald W. 2005. Involvement of *Phytophthora* spp. in the decline of European beech in Europe and the USA. Mycologist 19:159-166.

43. Jung T., and Blaschke H. 1996. *Phytophthora* root rot in declining forest trees. Phyton 36:95-102.

44. Kong P., Hong C. X., Richardson P. A., and Gallegly M. E. 2003. Single-strand-conformation polymorphism of ribosomal DNA for rapid species differentiation in genus *Phytophthora* . Fungal Genetics and Biology 39:238-249.

45. Kroon, L. P. N. M., Makker F. T., van den Bosch, G. B. M., Bonants P. J. M., and Flier W. G. 2004. Phylogenetic analysis of *Phytophthora* species based on mitochondrial and nuclear DNA sequences. Fungal Genetics and Biology 41:766-782.

46. Linderman R. G., Davis E. A., and Marlow J. L. 2006. Response of selected nursery crop plants to inoculation with isolates of *Phytophthora ramorum* and other *Phytophthora* species. Hort Technology 16:216-224.

47. Man in't Veld, Willem A., Veenbaas-Rijks W. J., Ilieva E., de Cock, Arthur W. A. M., Bonants P. J. M., and Pieters R. 1998. Natural hybrids of *Phytophthora nicotianae*

and *P. cactorum* demonstrated by isozyme analysis and random amplified polymorphic DNA. Phytopathology 88:922-929.

48. Martin F. N., and Tooley P. W. 2003. Phylogenetic relationships among *Phytophthora* species inferred from sequence analysis of mitochondrially encoded cytochrome oxidase I and II genes. Mycologia 95:269-284.

49. Matheron M. E., and Mircetich S. M. 1985. Seasonal variation in susceptibility of *Juglans hindsii* and paradox rootstocks of English walnut trees to *Phytophthora citricola*. Phytopathology 75:970-972.

50. Motta W., and Annesi T. 2003. A new *Phytophthora* sp. causing a basal canker on beech in Italy. Plant Disease 87:1005.

51. Oßwald W., Koehl J., Heiser I., Nechwatal J., and Fleischmann F. 2004. New insights in the genus *Phytophthora* and current diseases these pathogens cause in their ecosystem. Progress in Botany 65:436-466.

52. Oudemans P., Forster H., and Coffey M. D. 1994. Evidence for distinct isozyme subgroups within *Phytophthora citricola* and close relationships with *P. capsici* and *P. citrophthora* . Mycological Research 98:189-199.

53. Posada D., and Crandall K. A. 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14:817-818.

54. Ronquist F., and Huelsenbeck J. P. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19:1572-1574.

55. Sewell G. W. F., Wilson J. F., and Dakwa J. T. 1974. Seasonal variations in activity in soil of *Phytophthora cactorum*, *Phytophthora syringae* and *Phytophthora*

citricola in relation to collar rot disease of apple. Annals of Applied Biology 76:179-186.

56. Smith D. R., and Stanosz G. R. 1995. Confirmation of two distinct populations of *Spaeropsis sapinea* in the north central United States using RAPDs. Phytopathology 85:699-704.

57. Stamps D. J., Waterhouse G. M., Newhook F. J., and Hall G. S. 1990. Revised tabular key to the species of *Phytophthora*. Mycological Papers 162:1-28.

58. Swofford D. L. 2003. PAUP*: phylogenetic analysis using parsimony (*and other methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.

59. Tay F. C. S., Nandapalan K., and Davison E. M. 1983. Growth and zoospore germination of *Phytophthora* spp. on P10VP agar with hymexazol. Phytopathology 73:234-240.

60. Taylor J. W., Jacobson D. J., Kroken S., Kasuga T., Geiser D. M., Hibbett D. S., and Fisher M. C. 2000. Phylogenetic species recognition and species concepts in fungi. Fungal Genetics and Biology 31:21-32.

61. Tooley P. W., and Kyde K. L. 2007. Susceptibility of some eastern forest species to *Phytophthora ramorum*. Plant Disease 91:435-438.

62. Vettraino A. M., Barzanti G. P., Bianco M. C., Ragazzi A., Capretti P., Paoletti E., Luisi N., Anselmi N., and Vannini A. 2002. Occurence of *Phytophthora* species in oak stands in Italy and their association with declining oak trees. Forest Pathology 32:19-28.

63. Vettraino A. M., Natili G., Anselmi N., and Vannini A. 2001. Recovery and pathogenicity of *Phytophthora* species associated with a resurgence of ink disease in *Castanea sativa* in Italy. Plant Pathology 50:90-96.

64. Weste G., and Vithanage K. 1979. Production of sporangia by *Phytophthora cinnamomi* in forest soils. Australian Journal of Botany 27:693-701.

65. White T. J., Bruns T., Lee S., and Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pages 315-322 in: Innis, M. A., Gelfand, D. H., Sninsky, J. J., White T. J. eds. PCR Protocols: A guide to Methods and Applications. Academic Press Inc., San Diego, California.

CHAPTER 3

SUSCEPTIBILITY OF EUROPEAN BEECH (*FAGUS SYLVATICA*) AND OTHER WOODY HOSTS TO *PHYTOPHTHORA CITRICOLA* AND *P. CACTORUM* USING MULTIPLE INOCULATION METHODS

Abstract

Phytophthora cactorum and two distinct clades of *P. citricola* (*P. citricola* A and B) are associated with bleeding cankers on European beech (*Fagus sylvatica*) throughout the northeastern United States. Given the wide and diverse host range reported for these pathogens and the relatively uneven distribution of European beech in landscapes in the region, we hypothesized that the pathogens were playing a role in forest and landscape ecology beyond that of causing disease on one host species. In order to confirm the ability of isolates from *F. sylvatica* to cause disease on other plants, nine additional hosts were selected. Detached leaves, attached leaves, and living stems were inoculated with selected isolates of each taxon in order to determine susceptibility of different species and different types of tissues. Lesions developed as dark necrotic areas on leaf tissue, although not all plant-pathogen combinations resulted in disease development, and the aggressiveness of each pathogen varied. Stem lesions developed as necrotic or sunken regions. In general, stem tissue was less susceptible than foliage, with lesions developing on only 4 of the 10 plant species tested. Overall, we found that *P. cactorum, P. citricola* A and *P. citricola* B can infect leaves and stems of multiple plant species. Therefore, these pathogens have the potential to be infecting additional plant species in the forest and landscape environment beyond European beech. The symptom expression observed in this study

will enable surveyors in the field to better evaluate these potential host/pathogen relationships.

Introduction

Bleeding canker is a frequent and occasionally lethal disease of European beech in the northeastern United States (Chapter 2). Several pathogenic microorganisms are able to cause this disease, the most common being *Phytophthora cactorum* and *P. citricola*. These pathogens are relatively common on European beech and in the soil in their rhizospheres (Chapter 2). In addition, they have often been reported in orchards, nurseries and greenhouses (7, 8). However, their occurrence and distribution among native plant species is less well documented.

Phytopthora cactorum and *P. citricola* have wide host ranges, with 65 and 15 susceptible genera reported from North America, respectively (2). However, it is unclear whether isolates from specific hosts are able to cause disease on many plant species, or if they exhibit some amount of host specificity. For example, research into intraspecific diversity of *P. citricola* by Oedemans *et al* (10) showed that isolates from avocado had distinct phenotypes and isozyme patterns, suggesting that groups of these pathogens may be host-specific and genetically isolated. *Phytophthora citricola* has been shown to have a high amount of diversity, and recent research suggests that there are at least two distinct phylogenetic groups of *P. citricola* (denoted *P. citricola* A and *P. citricola* B) associated with bleeding canker of beech that may be better described as two distinct species (3, 7, chapter 2). Hong *et al* (7) found that isolates from one clade of *P. citricola* had a broader host range than previously thought. Knowledge of the ability of these pathogens to infect other plants is important as we try to determine how the pathogen spreads across long distance (sometimes hundreds of km) to get from one beech to another.

We hypothesize that individuals of *P. cactorum*, *P. citricola* A and *P. citricola* B from European beech are able to cause disease on at least several, perhaps many, native plant species. Furthermore, we suspect this heretofore underestimated diversity of potential hosts helps to account for some of the long distance spread of *Phytophthora* spp. between beech. In order for the beech pathogens to spread from tree to tree, it seems likely that other plant species are acting as intermediate hosts. This model of disease development is similar to that occurring in the case of forest tree and shrub diseases caused by *P. ramorum* (6). If the pathogens are being spread following this model, then *P. cactorum*, *P. citricola* A and *P. citricola* B are playing a role beyond causing disease on European beech. Ultimately, field observations are needed in order to confirm these assumptions, but right now we are not sure just what symptoms these pathogens might be causing and therefore what symptoms to look for. The species in question are known to cause disease of roots, stems and leaves, and of infecting young and mature plants (2). In order to determine what hosts are likely candidates and what type of symptoms they might exhibit, we conducted inoculation tests on greenhouse grown plants.

Artificial inoculations have been used in the past in order to determine host range of *Phytophthora* species on woody plants, and most recently they have helped to determine host range for *P. ramorum*. With *P. ramorum*, detached, attached, stem and log inoculations have shown that many more plants species than originally thought were hosts of *P. ramorum* and the focus on the species shifted from its role as a cause of bleeding cankers on oak to a cause of widespread foliar diseases (1, 4, 5, 11, 12). Similar studies have focused on other *Phytophthora* spp, including *P. cactorum* and *P. citricola* (7, 9). These studies found differing levels of susceptibility depending on the species of *Phytophthora* as well as the plant species. While artificial inoculations

introduce multiple factors not found in natural systems, Hansen *et al* (5) concluded artificial inoculations and natural infection correspond well.

The objectives of this research were (i) to evaluate the aggressiveness of *P. cactorum* and both groups of *P. citricola* from European beech on other recorded hosts of these pathogens, (ii) to assess the ability of these pathogens to infect leaf and stem tissue and (iii) to determine what symptoms might look like as we commence surveys in natural systems.

Methods

Plant and Pathogen material

Isolates of *Phytophthora* were obtained by culturing from diseased European beech and soil as described previously (Chapter 2) and were maintained on corn meal agar (Table 3.1). Potential plant hosts were selected based on four criteria: (i) published reports of susceptibility (2), (ii) frequency of the plant in the landscapes of the northeastern United States, (iii) suitability for cultivation, and (iv) confirmed susceptibility based on preliminary detached leaf studies (data not shown). Using these criteria, ten plant species were selected for testing, including European beech (Table 3.2). Plant material was obtained by growing seedlings from seed collected on the Cornell campus or purchased from a commercial firm (F. W. Schumacher Co., Sandwhich, MA) or from seedlings provided by Lawyer Nursery (Plains, MT) (Table 3.2). Leaves were collected from trees in the Ithaca area during May and June when the leaves were fully expanded. Leaves collected from plants in the greenhouse were also fully expanded. Plants were tested for susceptibility using detached leaf, attached leaf and stem inoculations.

Table 3.1. Isolates used for inoculation studies.

Isolate	Species
NYfs20	*P. cactorum*
NYfs24	*P. cactorum*
NYfs11	*P. citricola* A
MDfs1	*P. citricola* A
NYfs18	*P. citricola* A
NYas1	*P. citricola* B
NYfs9	*P. citricola* B

Table 3.2. Plant host list. Source of plant material is listed for each inoculation method.

Host	Common Name	Source	Inoculations
Fagus sylvatica	European beech	Seeds from Schumacher	Stem, attached
		Trees in Ithaca	Detached leaf
Syringa vulgaris	common lilac	Seed collected in Ithaca	Stem, attached, detached
Acer saccharum	sugar maple	Lawyer nursery seedlings	Stem, attached
		Trees in Ithaca	Detached
Betula lenta	sweet birch	Lawyer nursery seedlings	Stem, attached
		Trees in Ithaca	Detached
Ulmus americana	American elm	Lawyer nursery seedlings	Stem, attached, detached
Viburnum trilobum	American cranberry bush	Lawyer nursery seedlings	Stem, attached
		Trees in Ithaca	Detached
Viburnum opulus	European cranberry bush	Trees in Ithaca	Detached
		Lawyer nursery seedlings	Stem, attached
Viburnum dentatum	arrowwood viburnum	Lawyer nursery seedlings	Stem, attached
		Leaves collected in Ithaca	Detached
Fraxinus americana	white ash	Lawyer nursery seedling	Attached, stem
		Trees in Ithaca	Detached
Syringa reticulata	tree lilac	Seeds collected in Ithaca	Stem, attached, detached

Detached leaf inoculations

A 15 cm diameter glass petri plate containing a 15 cm diameter piece of filter paper was autoclaved, and then distilled water was added to each dish until the filter paper was saturated. Leaves were removed from plants and within 30 minutes they were placed abaxial surface up in the petri dish. Inoculation points were scratched lightly 5 times with an insect pin in order to etch the cuticle without perforating the leaf. One 5 mm plug was taken from the margin of a 7 day old colony of the pathogen growing on clarified V8 agar (2) and placed mycelium side down onto the wounded area of the leaf. Control inoculations used sterile clarified V8 (cV8) agar plugs. The number of inoculations varied from 1 to 5 per leaf depending on leaf size. Three days after inoculation, leaves were evaluated for symptom development and a subset with symptoms was used for reisolation. Leaves were rated for the presence of necrosis as well as the size of the lesion. Each host-isolate combination was repeated 10 times and this experiment was conducted twice.

Attached leaf inoculations

Isolates were grown on cV8 agar for 1-2 weeks. Fifteen mm diameter plugs from the margin of the colony were then transferred to a new Petri dish and flooded with approximately 20 ml of 10% filtered and autoclaved soil extract water (2). Two days later, the water was drained and the plugs were reflooded. After another two days, the presence of sporangia was confirmed visually and the plates were chilled at 4ºC for 30 minutes. Plates were returned to room temperature and then checked again visually to confirm that zoospores had been released. The number of zoospores was estimated using a hematocytometer and each solution was diluted, using soil extract water, to 5000 zoospores per ml.

Plants were inoculated by stapling a 7 mm diameter filter paper disk to the approximate center of one side of the leaf blade (or the center of the lobe in palmately

leaved plants). A 50 μl aliquot of the zoospore suspension was then placed under the filter disk. Control inoculations were conducted by using soil extract water from a dish with sterile agar plugs. The number of inoculation points per plant varied due to differences in the gross morphology of the plants being inoculated. In cases where each plant had multiple stems (all three *Viburnum* species, *Betula lenta* and *Ulmus americana*) two leaves on one branch were inoculated with one isolate and then two leaves on a separate branch were inoculated with a different isolate. If a plant had only one stem (*Acer saccharum*, *Syringa reticulata*, *S. vulgaris* and *F. sylvatica*) then only two leaves on the plant were inoculated, each with the same isolate. For *Fraxinus americana*, a compound leaf was inoculated twice, on separate leaflets, with the same isolate, and two leaves per plant were inoculated, each with a different isolate. The leaf, branch or plant was then placed in a plastic bag and sprayed with distilled water to runoff. Plants were left in the bag for seven days, and resprayed daily to runoff. After one week, the bag was removed and symptoms were recorded by indicating the presence or absence of a lesion, the size of the lesion, the persistence of the leaf on the stem, and progression from leaf to stem infection. In addition, reisolation was attempted from a subset of the plants that developed symptoms. Two trials were conducted, with each trial having 4 replicates of each host-isolate combination.

Stem inoculations

Cultures were grown on cV8 agar for 7-10 days and then a 5 mm diameter plug was removed from the margin of each colony. Using a 5 mm diameter cork borer, bark was removed from the main stem of the plant and the agar plug, mycelium side in, was placed in the hole. For a control, uncolonized V8 agar plugs were used. Parafilm was wrapped around the branch to cover the wound. After 2 weeks the parafilm was removed and the plant was checked visually for symptom development.

If necrosis was present, lesions were measured in the vertical direction and also for percent of stem girdle. A subset of plants that developed symptoms was sampled to ensure that we could reisolate the pathogen and to confirm that size of the external canker corresponded with the size of the area of necrosis found within the bark. Two trials were conducted, and each trial had 3 replicates of each host-pathogen combination.

Data Analysis

In all inoculation methods, the presence or absence of a lesions was recorded, and lesion severity was rated (1= no lesion, 2= up to 10 mm, 3=11-20 mm, 4= 20 + mm) based on a system developed by Linderman *et al* (9). This system allowed for comparison between lesions regardless of constrictions due to leaf or stem size. Frequency of lesion incidence, leaf detachment, and infection spreading from a leaf to a stem were calculated. Differences between the severity ratings for each host plant/pathogen species combination were compared using nonparametric tests analogous to analysis of variance (procedure npar1way in SAS). All possible pairwise comparisons were conducted using the stepboot option in the multtest procedure (SAS, version 9.1 for Windows, SAS Institute, North Carolina, USA) with 50000 replicates (9).

Results

All three inoculation methods resulted in the development of necrosis around the inoculation point in at least some instances. Necrosis in attached leaf and detached leaf inoculations was similar in that both methods produced dark water-soaked lesions that expanded out from the point of inoculation. Symptoms would sometimes progress more rapidly along the axis parallel to the midrib of the leaf, but were not limited by veins. Detached leaf inoculations produced symptoms for all three

pathogens on all host plants in at least some instances (ranging from 25-100% depending on host and pathogen species) with the exception of *P. cactorum* on *S. reticulata*, *V. opulus* and *V. dentatum* (Figure 3.1).

Attached leaf inoculations produced symptoms in at least some instances (ranging from 17-100% depending on host and pathogen species) for all three pathogens on all hosts with the exception of *V. dentatum*, which never had symptoms regardless of pathogen species, and *S. reticulata* which only developed symptoms when inoculated with *P. citricola* A (Figure 3.2). Attached leaf inoculations allowed for the additional observation of persistence of leaf attachment. *Fagus sylvatica* and *S. reticulata* did not have any instances of leaf detachment (n=60 for each host plant). All other host plants dropped inoculated leaves in at least one instance (ranging from 4-56% depending on host and pathogen species) during the 7 day trial when inoculated with each of the three pathogen species except for *P. cactorum* on birch and *P. citricola* A on *V. dentatum*. Control inoculations never caused detachment of leaves with the exception of *V. opulus* (Figure 3.3). In addition, attached leaf inoculations allowed for observation of progression of the pathogens from leaves to stems. This occurred with varying frequency for all three pathogens in *F. sylvatica* and *S. vulgaris*, but only for *P. cactorum* and *P. citricola* B on *B. lenta* (Figure 3.4).

Figure 3.1. Detached leaf lesion incidence and severity. Incidence expressed as a percentage, average severity based on lesion diameter size (1= no lesion, 2= up to 10 mm, 3=11-20 mm, 4= 20 + mm). Number of inoculations per host plant species varies by pathogen (*P. cactorum* n=40, *P. citricola* B n=40, *P. citricola* A n=60). For severity, means with the same letter were not significantly different at alpha=0.5 based on the nonparametric test for comparing means.

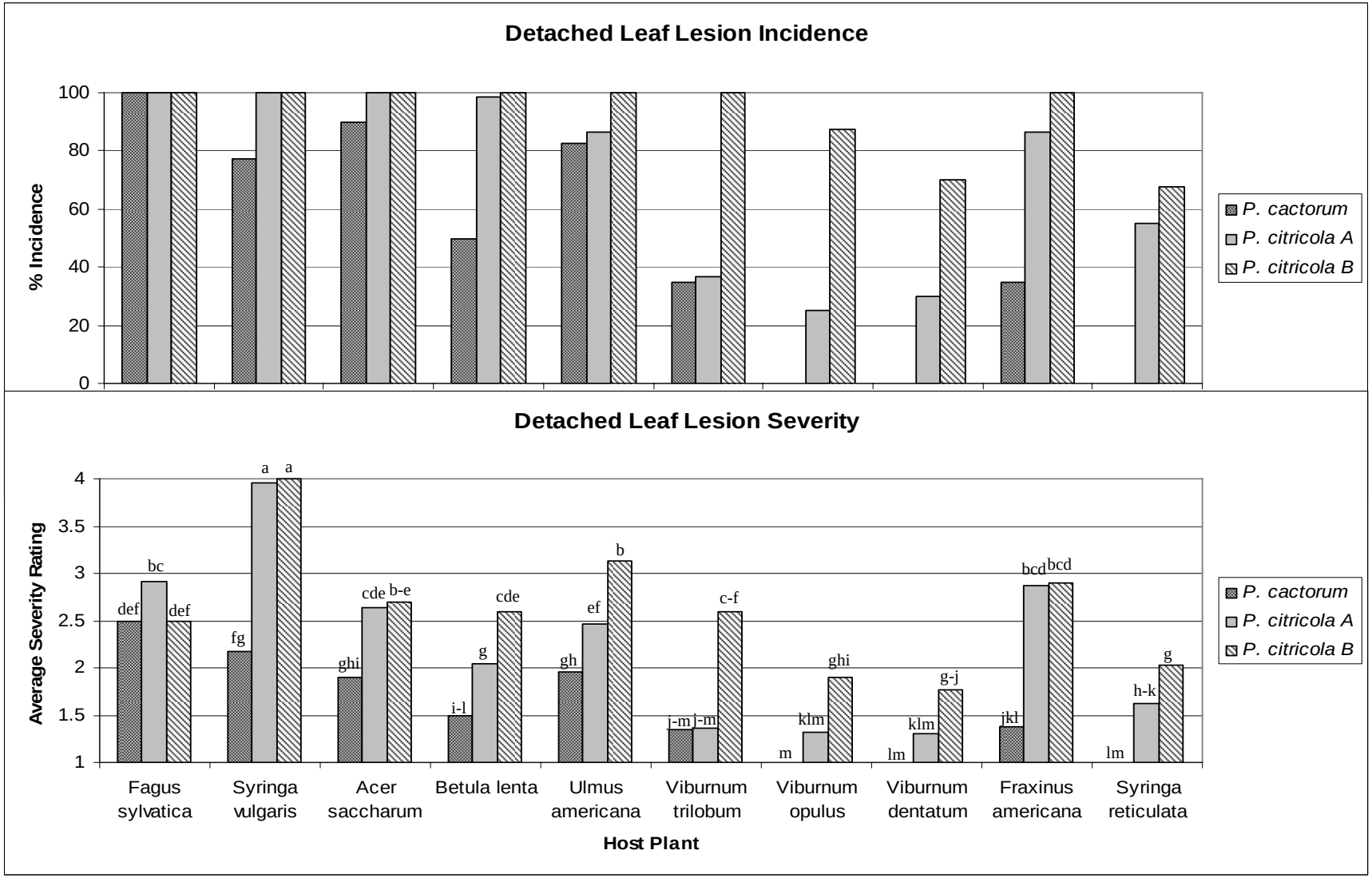
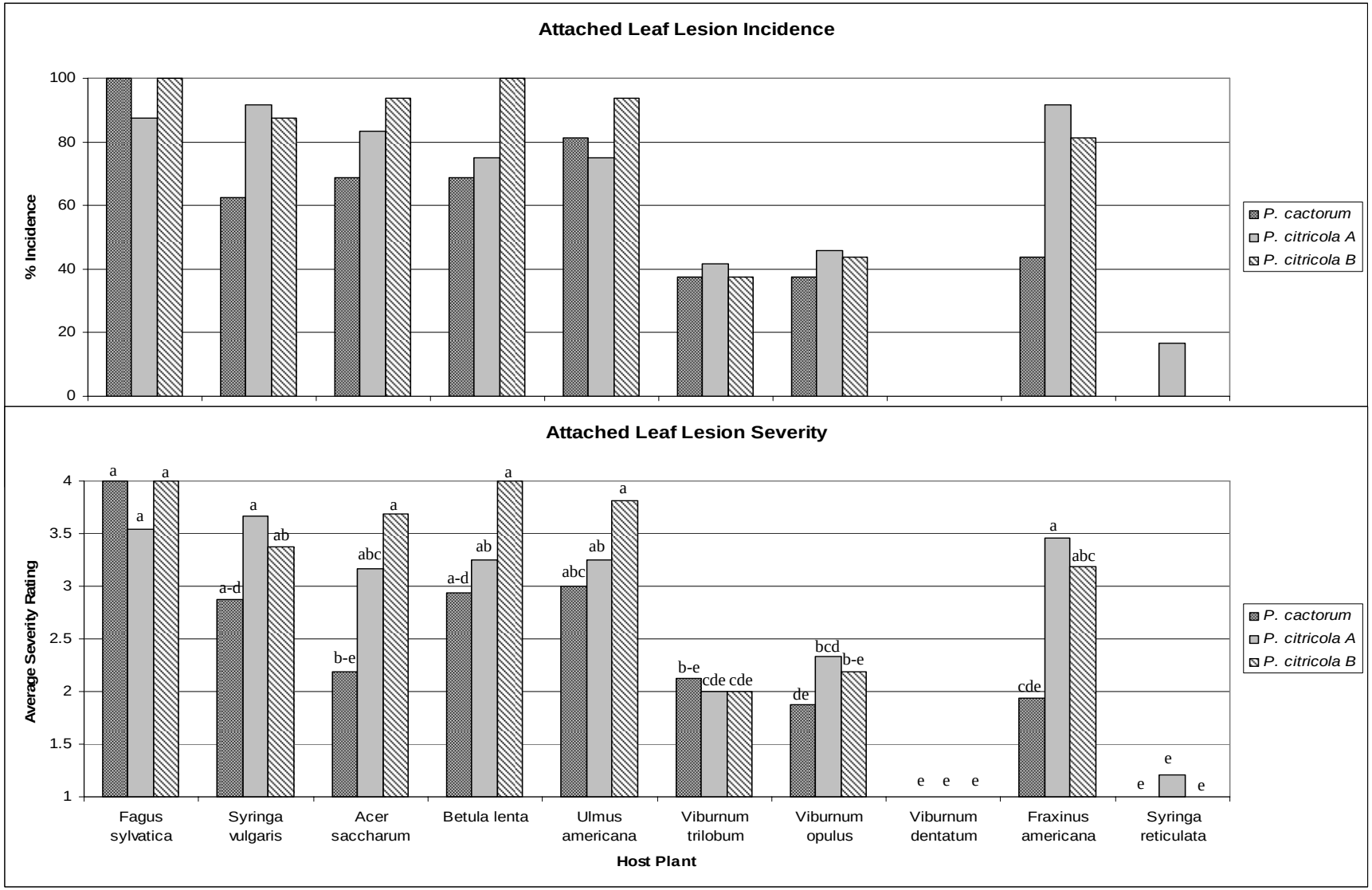

Detached Leaf Lesion Incidence
% Incidence
100
80
60
40
20
0
P. cactorum
P. citricola A
P. citricola B
Detached Leaf Lesion Severity
Average Severity Rating
4
3.5
3
2.5
2
1.5
1
def bc def
fg a a
ghi cde b-e
i-l g cde
gh ef b
j-m j-m c-f
m klm ghi
lm klm g-j
jkl bcd bcd
lm h-k g
Fagus sylvatica
Syringa vulgaris
Acer saccharum
Betula lenta
Ulmus americana
Viburnum trilobum
Viburnum opulus
Viburnum dentatum
Fraxinus americana
Syringa reticulata
Host Plant

Figure 3.2. Attached leaf lesion incidence and severity. Incidence expressed as a percentage, average severity based on lesion diameter size (1= no lesion, 2= up to 10 mm, 3=11-20 mm, 4= 20 + mm). Number of inoculations per host plant species varies by pathogen (*P. cactorum* n=16, *P. citricola* B n=16, *P. citricola* A n=24). For severity, means with the same letter were not significantly different at alpha=0.5 based on the nonparametric test for comparing means.


Attached Leaf Lesion Incidence
% Incidence
100
80
60
40
20
0
P. cactorum
P. citricola A
P. citricola B
Attached Leaf Lesion Severity
Average Severity Rating
4
3.5
3
2.5
2
1.5
1
a a a
a-d a ab
b-e abc a
a-d ab a
abc ab a
b-e cde cde
de bcd b-e
e e e
cde a abc
e e e
Fagus sylvatica
Syringa vulgaris
Acer saccharum
Betula lenta
Ulmus americana
Viburnum trilobum
Viburnum opulus
Viburnum dentatum
Fraxinus americana
Syringa reticulata
Host Plant
P. cactorum
P. citricola A
P. citricola B

Stem inoculations caused the least amount of disease, with only four host species developing symptoms (Figure 3.5). In plants that did develop symptoms, the type of symptoms that developed differed. *Syringa vulgaris*, *F. sylvatica* and *B. lenta* developed necrotic lesions that extended out from the inoculation point and into the xylem tissue. In *U. americana*, lesions appeared as sunken areas. Excavation into the inoculation site indicated development of wound response tissue but not discoloration and necrosis. Control inoculations did not produce similar responses, but instead wound tissue developed without any sunken areas. *Fagus sylvatica*, *S. vulgaris* and *U. americana* developed stem symptoms for all three pathogen species, while *B. lenta* only developed symptoms for *P. citricola* A and B. All reisolation attempts from detached, attached and stem inoculations were successful.

Comparison of severity ratings using the nonparametric statistical test indicated significant differences. Comparisons were only made within each method of inoculation. Results varied by host and pathogen species. Average severity ratings are found in Figures 3.1, 3.2, and 3.5, with significant differences between severity ratings within each method indicated by different letters.

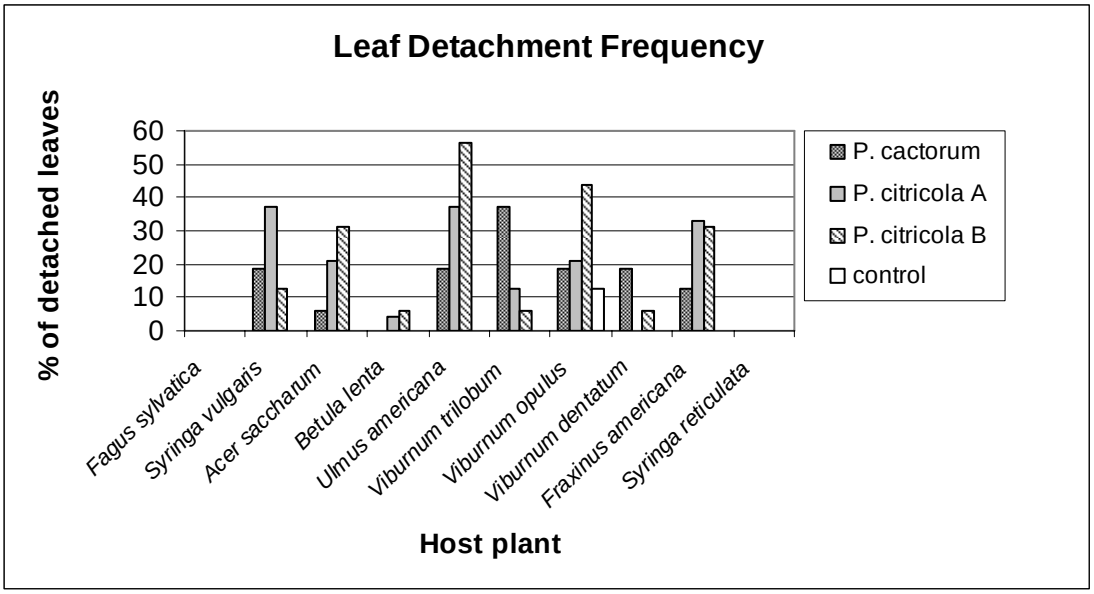


Figure 3.3. Leaf detachment frequency. Percentage of leaves that became detached for each plant/pathogen combination. Number of inoculations per host plant species varies by pathogen (*P. cactorum* n=16, *P. citricola* B n=16, *P. citricola* A n=24).

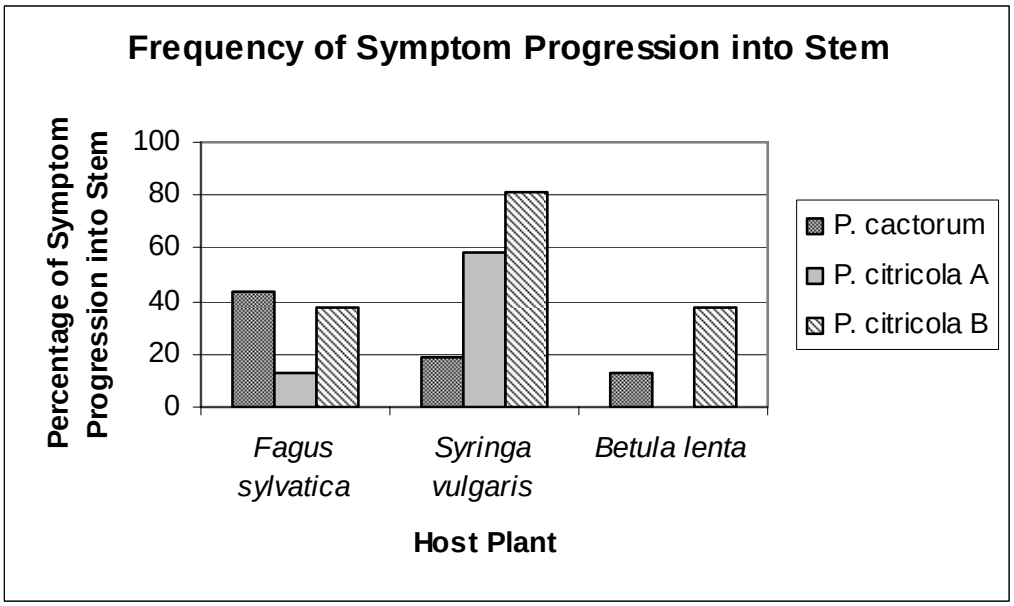
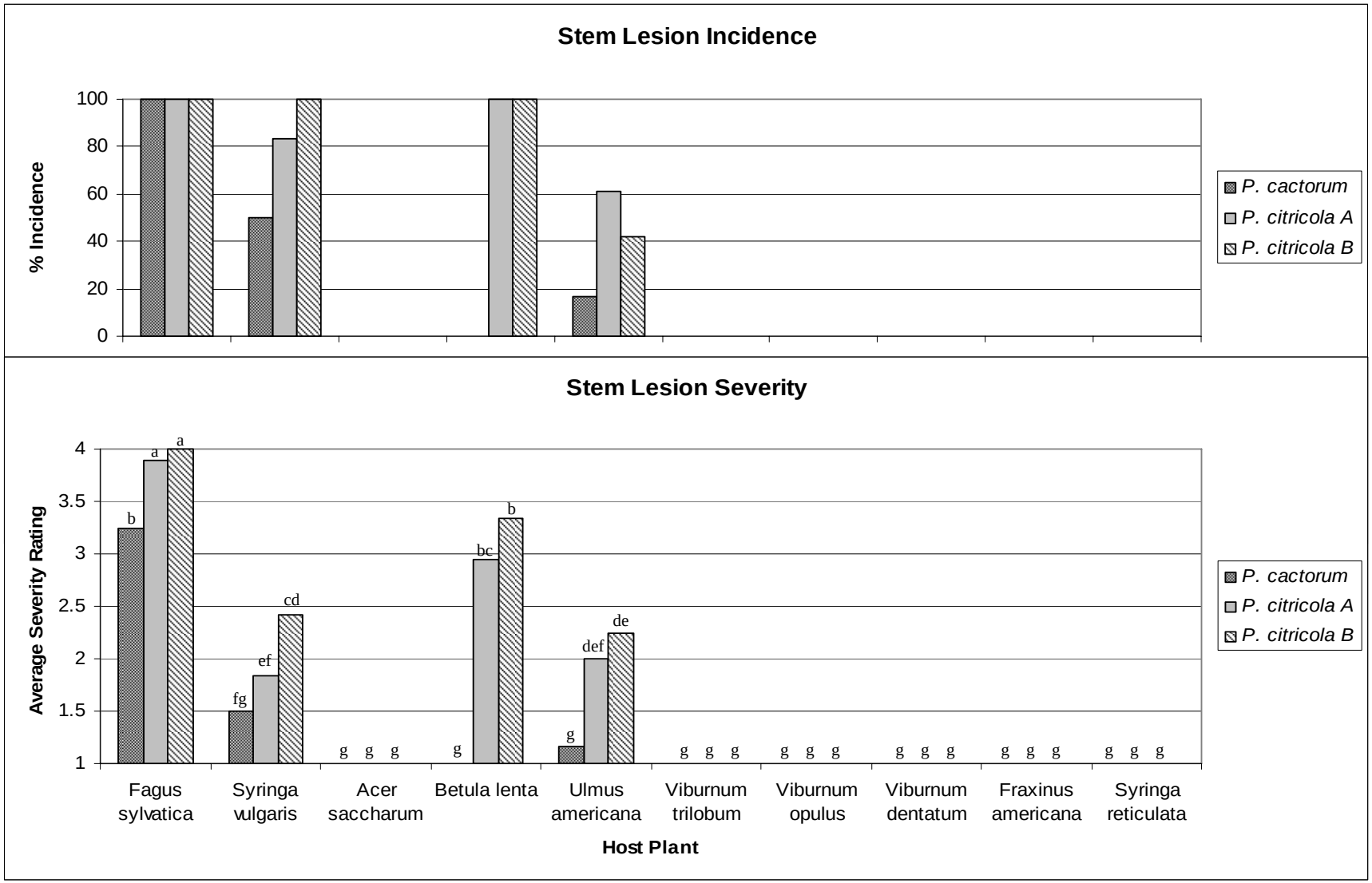


Figure 3.4. Progression of attached leaf infections into stem. Percentage of attached leaf inoculations that progressed into the stem. Host plants that did not develop symptoms in this way are not included. Number of inoculations per host plant species varies by pathogen (*P. cactorum* n=16, *P. citricola* B n=16, *P. citricola* A n=24).

Figure 3.5. Stem lesion incidence and severity. Incidence expressed as a percentage, average severity based on lesion diameter size (1= no lesion, 2= up to 10 mm, 3=11-20 mm, 4= 20 + mm). Number of inoculations per host plant species varies by pathogen (*P. cactorum* n=12, *P. citricola* B n=12, *P. citricola* A n=18).For severity, means with the same letter were not significantly different at alpha=0.5 based on the nonparametric test for comparing means.


Stem Lesion Incidence
% Incidence
100
80
60
40
20
0
P. cactorum
P. citricola A
P. citricola B
Stem Lesion Severity
Average Severity Rating
4
3.5
3
2.5
2
1.5
1
b
a
a
fg
ef
cd
g g g
g
bc
b
g
def
de
g g g
g g g
g g g
g g g
g g g
Fagus sylvatica
Syringa vulgaris
Acer saccharum
Betula lenta
Ulmus americana
Viburnum trilobum
Viburnum opulus
Viburnum dentatum
Fraxinus americana
Syringa reticulata
Host Plant

Discussion

Comparisons of aggressiveness between pathogen and methods of inoculation are difficult in this study due to the fact that the plants tested were not genetically identical, and therefore differential responses could be the result of differences in host resistance. Differing levels of resistance have been observed in *Fagus sylvatica* seedlings infected with *P. citricola* and *P. cactorum* (14). It is likely that there are differing levels of resistance within all of the host plants tested. While this may make patterns more difficult to observe by introducing additional variation, some important trends were still apparent.

Phytophthora cactorum, *P. citricola* A and *P. citricola* B all caused disease on other plant species in addition to *F. sylvatica*. Given the wide host range reported for *P. cactorum* and *P. citricola* (2) these results are not surprising. However, because two distinct clades of *P. citricola* were used, it was unclear if there might be some host specificity involved. This has been demonstrated for groups of isolates of *P. citricola* in the past (10). Based on our results, both groups of *P. citricola* appear to be able to cause disease on a range of plant hosts.

Detached leaf inoculations resulted in much higher disease incidence than attached leaf or stem inoculations. This suggests that detached leaf inoculations may overestimate susceptibility of a host, particularly in regard to attached leaf inoculation responses. However, other factors may have affected the differences seen between these two inoculation methods. The detached leaf method included scratching to wound the leaves, and then agar plugs were used as inoculum. Attached leaf inoculations, on the other hand, included use of a staple to create a wound and then a zoospore suspension on filter paper discs as inoculum. This method was adopted because of the difficulty in confining inoculum to a particular site on a leaf that was still attached to a plant. The presence of the filter paper disk at the site of the wound

had a sponge-like effect, holding the moisture, and the zoospores, at the desired infection site. While this protocol allowed us to inoculate attached leaves, it introduced other possible factors that may have changed disease incidence for attached leaf inoculations as compared to detached leaf inoculations.

An additional factor creating differences between detached and attached leaf inoculation disease incidence may have been the age of the leaves. Hansen *et al* (5) compared susceptibility of young and mature tanoak (*Lithocarpus densiflorus*) and myrtlewood (*Umbellularia californica*) leaves to *Phytophthora ramorum* zoospore suspensions and found that young leaves were more susceptible. In this study, detached leaf inoculations were conducted on fully expanded but recently emerged leaves, while attached leaf inoculations were conducted on older leaves. This may also account in part for the higher disease incidence seen in detached leaf inoculations.

Bleeding cankers are observed on the root flares and trunks of European beech, and infection of leaves has not been observed. However, detached leaf and attached leaf inoculations are often used to assay for host susceptibility, even when the disease being studied is found on the stems of plants (1, 5, 9, 11, 12). Sometimes these methods correspond with disease in natural settings (5) and we believe that our inoculations provide us with a good starting point for additional field surveys.

Leaf inoculation methods had added value in our study because they indicate how leaf symptoms might appear in naturally occurring disease. Although low levels of leaf infections might not have a high impact on deciduous plants, particularly if the infection occurs on leaves that are already detached, it does have an ecological impact if these infections are acting as sources of inoculum for other plants. Subsequent studies looking for confirmation of these host ranges in nature can use the type of symptoms seen here as an indication of what to look for.

Leaf infections that progress down the petiole and into the stem can also have a larger impact on host plant health. This type of symptom progression has been observed in leaf infections caused by *P. ramorum*, *P. cactorum*, *P. citricola*, *P. citrophthora*, and *P. nicotianae* (5, 9), and in this study it was observed in *B. lenta*, *S. vulgaris*, and *F. sylvatica*. These three plants also developed symptoms from stem inoculations; thus the ability of infection to spread from leaves to stem may be an indicator of general stem susceptibility.

The two different types of stem symptoms that developed suggest that as we search for confirmation of host range through identification of disease in the field, we should consider both necrotic lesions, as seen on *F. sylvatica*, as well as sunken areas, such as those seen on *U. americana*. Conversely, leaf symptoms did not seem to vary between hosts.

Phytophthora cactorum seems to be less aggressive than the other two pathogens. The isolates used in this study did not cause disease on *S. reticulata* or *V. dentatum*. This corresponds with previous studies indicating that *P. cactorum* was less aggressive than *P. citricola* A or B (Appendix A, 13, 14). Interestingly, although stem inoculations with *P. cactorum* did not cause any symptoms on *B. lenta*, stem infections did develop from attached leaf infections in some cases. This would seem to indicate that *P. cactorum* is capable of infecting *B. lenta* stems. Conversely, *P. citricola* A did cause stem cankers but attached leaf infections did not progress into the stem.

The ability of these pathogens to infect both leaves and stems of additional host plants suggests the possibility that other plants may be acting as sources of inoculum in a *Phytophthora* disease cycle of landscapes and forests. This model of disease development, described by Hansen (6) is new for *Phytophthora* as a forest pathogen and is typified by *P. ramorum*. If subsequent field studies show that

additional plant species are hosting *P. cactorum*, *P. citricola* A and *P. citricola* B, then bleeding canker of European beech could be developing as inoculum produced on leaves and stems of other plants is wind blown or splash dispersed onto the trunks of European beech. This type of spread of inoculum has very different management strategies and therefore it is important to continue to pursue this line of study by determining if these putative host plants do indeed host infections in natural settings.

REFERENCES

1. Denman S., Kirk S. A., Brasier C. M., and Webber J. F. 2005. *In vitro* leaf inoculation studies as an indication of tree foliage susceptibility to *Phytophthora ramorum* in the UK. Plant Pathology 54:512-521.

2. Erwin D. C., and Ribeiro O. K. 1996. *Phytophthora* diseases worldwide. APS Press, St. Paul, Minn.

3. Gallegly M. E., and Hong C. X. 2008. *Phytophthora*: Identifying species by morphology and DNA fingerprints. APS Press, St. Paul MN.

4. Garbelotto M., Davidson J. M., Ivors K., Maloney P. E., Huberli D., Koike S. T., and Rizzo D. M. 2003. Non-oak native plants are main hosts for sudden oak death pathogen in California. California Agriculture 57:18-23.

5. Hansen E. M., Parke J. L., and Sutton W. 2005. Susceptibility of Oregon forest trees and shrubs to *Phytophthora ramorum*: a comparison of artificial inoculation and natural infection. Plant Disease 89:63-70.

6. Hansen E. M. 2008. Alien forest pathogens: *Phytophthora* species are changing world forests. Boreal Environment Research 13:33-41.

7. Hong C., Richardson P. A., and Kong P. 2008. Pathogenicity to ornamental plants of some existing species and new taxa of *Phytophthora* from irrigation water. Plant Disease 92:1201-1207.

8. Jeffers S. N., Aldwinckle H. S., Burr T. J., and Arneson P. A. 1982. *Phytophthora* and *Pythium* species associated with crown rot in New York apple orchards. Phytopathology 72:533-538.

9. Linderman R. G., Davis E. A., and Marlow J. L. 2006. Response of selected nursery crop plants to inoculation with isolates of *Phytophthora ramorum* and other *Phytophthora* species. Hort Technology 16:216-224.

10. Oudemans P., Forster H., and Coffey M. D. 1994. Evidence for distinct isozyme subgroups within *Phytophthora citricola* and close relationships with *P. capsici* and *P. citrophthora* . Mycological Research 98:189-199.

11. Tooley P. W., Kyde K. L., and Englander L. 2004. Susceptibility of selected ericaceous ornamental host species to *Phytophthora ramorum*. Plant Disease 88:993-999.

12. Tooley P. W., and Kyde K. L. 2007. Susceptibility of some eastern forest species to *Phytophthora ramorum*. Plant Disease 91:435-438.

13. Weiland J. E., Nelson A. H., and Hudler G. W. 2008. Aggressiveness of *Phytophthora cactorum* and *Phytophthora citricola* isolates on European beech and lilac. Phytopathology 98:S168-S169.

14. Werres S. 1995. Influence of the *Phytophthora* isolate and the seed source on the development of beech (*Fagus sylvatica*) seedling blight. European Journal of Forest Pathology 25:381-390.

CHAPTER 4

CONCLUSIONS

Bleeding canker of Euoropean beech is a common problem in the northeastern United States, where there are five pathogens involved. *Phytophthora cactorum* and *P. citricola* A are frequent, and *P. citricola* B, *P. gonapodyides* and *P. cambivora* are found rarely. These pathogens can be found in the environment surrounding European beech, in the rhizosphere and potentially on other plant hosts. The ability of these pathogens to persist either in soil or on other hosts provides multiple mechanisms for them to initiate disease.

Because all of the *Phytophthora* spp. isolated during our study had been previously described in the United States (4), it appears that the recent apparent increase in the occurrence of bleeding cankers on European beech is not due to the introduction of a new pathogen species such as *P. ramorum* or *P. kernoviae*. However, new genotypes of the pathogens with unique pathogenicity ranges could have been introduced. A population level study to explore the diversity of these species in the United States would help to determine if a newly invasive genotype or race has been introduced.

Increased disease incidence could also be due to changes in the environment that cause more stress to the trees. For example, severe drought conditions have been correlated with increased mortality in American beech in Maine (9) and increased incidence of bleeding cankers on oak in West Virginia (13). Changes in climate patterns have also been hypothesized to affect European oak forests (1). Similar environmental stresses could be playing a role in European beech health. Another possible cause in increased host susceptibility has been suggested by the observation

that a small number of trees in our survey had cankers that were centered on bark beetle entrance/exit holes. Individuals collected from infested bark were identified as *Euwallacea validus*, a bark beetle that was introduced into the United States from Asia in 1975 (6). Therefore, increased incidence of insect colonization over the last 30 years could have increased European beech susceptibility to infection by *Phytophthora spp.* However, because these beetles are attracted to stressed or diseased trees, increased colonization may be a result, and not a cause, of increased disease incidence. However, the role of these or other insects in the epidemiology of *Phytophthora* spp. on beech is unknown, and we cannot rule out a possible interaction that would lead to an increase in disease incidence.

Another possibility is the phenomenon of synchronous cohort decline, described by Mueller-Dumbois *et al* (10) to account for death of large populations of trees of the same age in a relatively discrete geographic region. In this model, trees become susceptible to a pathogen at approximately the same time because they are all the same age. European beech is a common landscape tree but it was especially popular for planting from about 1870-1940 (12). Many of the trees surveyed in these studies were planted during that time. However, symptoms were seen on trees younger than that, so further testing is needed in order to establish the relationship between tree age and susceptibility.

Human perception may also be influencing the apparent increase in the disease. The public's awareness of bleeding canker as an important disease symptom has increased due to media coverage of diseases such as sudden oak death, or ramorum blight, caused by *P. ramorum*. As people are more aware of this symptom, they may be more likely to notice it and report it to arborists or other tree care professionals. One or more of these factors may be acting to cause an increase in reports of bleeding cankers on European beech.

There are multiple models for disease development for *Phyophthora* spp. One commonly held model is that spread and sporulation occur in soil when plant exudates break dormancy of pathogen resting spores (11). Our research has shown that these pathogens are present and can be triggered to sporulate in the soil microenvironment (Chapter 2). Additional studies have shown that European beech seedlings and saplings are susceptible to root infection (Appendix A, 5, 8). However, our observations do not always support this disease development model, inasmuch cankers are sometimes located well above the soil line with no apparent symptom development in between.

Another more recently developed model states that these pathogens sporulate on aerial plant parts and spread through wind or rain splashing (7). Pathogen infection of leaf and stem tissue of other plants, as shown in Chapter Three, could then provide direct sources of inoculum. Other alternative explanations for symptom development far above the soil line include infection that occurs in the xylem without producing external symptoms, and then spreads to other parts of the plant, as described in Brown and Brasier (2) or insects or other vectors carrying propagules from soil to aboveground portions of the tree, as described in El Hamalawi and Menge (3). Further research is needed to explore all of these hypotheses.

Overall, the research reported here is consistent with disease development through soil infection or from sporulation on other host plants. Both development pathways could be playing a role in the case of bleeding canker of European beech. While the pathogens can be found in the soil surrounding healthy and symptomatic European beech, they also have the potential to infect other plants in the area. Because of the multi-faceted mode of disease development, management strategies need to include multiple methods to both prevent and treat diseased trees.

REFERENCES

1. Brasier C. M., and Scott J. K. 1994. European oak declines and global warming: a theoretical assessment with special reference to the activity of *Phytophthora cinnamomi*. EPPO Bulletin 24:221-232.

2. Brown A. V., and Brasier C. M. 2007. Colonization of tree xylem by *Phytophthora ramorum, P. kernoviae* and other *Phytophthora* species. Plant Pathology 56:227-241.

3. El Hamalawi Z. A., and Menge J. A. 1996. The role of snails and ants in transmitting the avocado stem canker pathogen, *Phytophthora citricola*. Journal of the American Society for Horticultural Science 121:973-977.

4. Erwin D. C., and Ribeiro O. K. 1996. *Phytophthora* diseases worldwide. APS Press, St. Paul, Minn.

5. Fleischmann F., Schneider D., Matyssek R., and Oßwald W. F. 2002. Investigations on net CO2 assimilation, transpiration and root growth of *Fagus sylvatica* infested with four different *Phytophthora* species. Plant Biology 4:144-152.

6. Haack R. A. 2001. Intercepted Scolytidae (Coleoptera) at U.S. ports of entry: 1985-2000. Integrated Pest Management Reviews 6:253-282.

7. Hansen E. M. 2008. Alien forest pathogens: *Phytophthora* species are changing world forests. Boreal Environment Research 13:33-41.

8. Jung T., Hudler G. W., Griffiths H. M., Fleischmann F., and Oßwald W. 2005. Involvement of *Phytophthora* spp. in the decline of European beech in Europe and the USA. Mycologist 19:159-166.

9. Kasson M. T. 2007. Relationships between climate, disease, radial growth response, and mortality of American Beech in Northern Maine. University of Maine, Maine.

10. Mueller-Dumbois D., Canfield J. E., Holt R. A., and Buelow G. P. 1983. Tree-group death in North American and Hawaiian forests: a pathological problem or a new problem for vegetation ecology? Phytocoenologia 11:137.

11. Oßwald W., Koehl J., Heiser I., Nechwatal J., and Fleischmann F. 2004. New insights in the genus *Phytophthora* and current diseases these pathogens cause in their ecosystem. Progress in Botany 65:436-466.

12. Pregill P., and Volkman N. 1999. Landscapes in history: design and planning in the eastern and western tradition. 2nd ed. John Wiley, New York.

13. True R. P., and Tryon E. H. 1956. Oak stem cankers initiated in the drought year 1953. Phytopathology 46:617-622.

APPENDICES

APPENDIX A

SEEDLING INOCULATION WITH INFESTED SOIL

European beech seedlings were planted in infested soil to determine if the pathogen had the ability to infect roots. Sixteen single zoospore isolates were selected from our collection to represent the geographic range of our isolates from European beech (Table A.1). Five isolates of *P. cactorum*, 8 isolates of *P. citricola* A and 3 isolates of *P. citricola* B were included. Inoculum was prepared by following the protocol described in Wilcox and Mircetich (9) with the following changes: the rice/v8 juice/vermiculite mixture was kept in 500 ml erlenmyer flasks and inoculated with two 10 mm plugs collected from the margin of the isolate growing on CMA. After four weeks, the flask was filled with water, the contents were stirred and then the water drained. Then, 20 ml of the resulting spawn were mixed with 1 L Cornell mix, a peatmoss and vermiculite soilless potting media (1).

European beech seedlings were grown in a greenhouse from seed (Sheffield Seed Co., Locke, NY) for 60-70 weeks. Just prior to exposure to the pathogens, seedlings were removed from 10 cm plastic pots, all excess Cornell mix was removed from the pot and the roots were rinsed to remove excess media. Trees were then replanted in the same pot but with the infested potting medium. Four trees were used per isolate, with one plant per pot. Uninoculated controls consisted of rice/v8 juice/vermiculite mixture with uncolonized agar plugs added. Immediately after potting and every two weeks following, each pot was placed in a plastic bag and flooded with tap water. After 48 hours, the plastic bags were removed and the pots were allowed to drain. Trees were monitored weekly for development of symptoms

such as discoloration of the stem, stem constriction and wilting of the crown. Symptomatic trees were destructively sampled for recovery of the pathogen to confirm infection by placing small (approximately 5 mm^2) chips of bark from the margin of the necrosis on PARP (2). After 6 months, all remaining plants were sampled for the presence of the pathogen by plating on selective media as described above. In addition, the soil from the remaining pots was checked for the pathogen using the baiting method described in Chapter 2. This experiment was conducted twice as an experimental repeat. The number of trees that displayed symptoms and yielded an isolate of *Phytophthora* was pooled for the two trials and then analyzed using the Kruskal-Wallis nonparametric test for significant differences in disease incidence between species of pathogen (Minitab Statistical Software, release 15.1; Minitab Inc., State College, PA).

Seventy-nine of the 128 inoculated trees developed symptoms and fifty-seven of those yielded *Phytophthora* when sampled. The percentage of plants killed by *Phytophthora* spp, or disease incidence, was defined as those plants that developed symptoms and yielded a *Phytophthora* isolate from the stem. This disease incidence varied depending on isolate, ranging from 0 to 87.5% (Table A.1). Soil infestation with *P. cactorum* resulted in death of 11 of 40 trees, *P. citricola* A resulted in 40 of 64 trees, and *P. citricola* B resulted in 6 of 24 trees. There were significant differences in mortality between species. *Phytophthora citricola* A was significantly different than *P. cactorum*, ($p=0.048$). Both *Phytophthora citricola* A and B and *P. citricola* B and *P. cactorum* were not significantly different ($p=0.083$ and $p=0.881$ respectively). While 42% of the test trees (57/128) did not develop symptoms, testing of the soil from these pots yielded *Phytophthora* in all but one case (56/57).

In general, these pathogens seem capable of infecting European beech through the roots. There was a high amount of variation between isolates but when isolates were grouped by taxa, there were some significant differences: *P. citricola* A was

more aggressive than *P. cactorum*. Additional aggressiveness studies have supported this difference (7).

Due to space and time constraints and availability of plant material, this experiment was conducted on European beech seedlings. Although the results provide some insight into susceptibility of seedlings to root infections, we have some reservations about their usefulness in making assumptions about infection courts on mature trees. Other plant species have been shown to change in susceptibility to *Phytophthora* spp. depending on age (6). However, the ability of seedlings to become infected through propagules in the soil does add support to the idea that the soil could be acting as a source of inoculum. In addition, it suggests an additional role that the pathogen may be playing in the environment: *Phytophthora* populations in the soil around mature trees may act to suppress the growth of seedlings around the parent tree. This has been documented for soil pathogens of *Prunus serotina* (5).

Table A.1. Isolates used for soil infestation study. Percentage mortality of European beech seedlings planted in infested soil is listed by isolate (n=8) and by species. Standard deviations are included for species totals. Letters indicate significant differences based on the Kruskal-Wallis nonparametric test.

Isolate name	Species	Location	Host	Percentage mortality in soil infestation
NCrh5	*P. citricola* A	North Carolina	Rhododendron	0
NYfs11[a]	*P. citricola* A	New York	European beech	87.5
MDfs2	*P. citricola* A	Maryland	European beech	75
NYfs22	*P. citricola* A	New York	European beech	62.5
MAfs3	*P. citricola* A	Massachusetts	European beech	75
MDfs1	*P. citricola* A	Maryland	European beech	75
PAfs2	*P. citricola* A	Pennsylvania	European beech	87.5
NYfs18	*P. citricola* A	New York	European beech	37.5
Average mortality for *P. citricola A*				**67.19 ± 31.99 a**
NYas2[a]	*P. citricola* B	New York	Sugar maple	12.5
NYfs9	*P. citricola* B	New York	European beech	25
NYso1	*P. citricola* B	New York	Soil	37.5
Average mortality for *P. citricola B*				**25 ± 12.5 ab**
MAfs2	*P. cactorum*	Massachusetts	European beech	12.5
NYfs20	*P. cactorum*	New York	European beech	0
NYfs21	*P. cactorum*	New York	European beech	37.5
NYfs24[a]	*P. cactorum*	New York	European beech	25
NYfs23	*P. cactorum*	New York	European beech	62.5
Average mortality for *P. cactorum*				**30 ± 20.91 b**

[a] these isolates were also used in the soil persistence study (Appendix B)

APPENDIX B

PERSISTENCE IN THE SOIL

In order to test the ability of the pathogens to persist in the soil, three isolates (one isolate of *P. cactorum*, one of *P. citricola* A and one of *P. citricola* B) used in the soil infestation study (Table A.1) were selected to determine their ability to persist in the soil. Two types of media were tested, commercially available top soil and Cornell mix (1). Media were infested as described in Appendix A. We also tested the effect of the presence or absence of common lilac (*Syringa vulgaris*), a host susceptible to all three groups of *Phytophthora*. Lilacs were grown from seed collected on the Cornell campus (Ithaca, NY) and raised in 5 cm plastic pots before being transplanted to the infested media. Infested media, with and without lilac seedlings, were placed in 10 cm plastic pots in a greenhouse. Each pathogen-media-host presence/absence combination was repeated four times with the exception of the control pots. Control pots were only made once for each treatment combination, resulting in a total of 52 pots. Five sets of 52 pots were made so that soil could be tested at 5 time points post inoculation: 1, 3, 6, 9 and 12 months. Soil was tested for the presence of the *Phytophthora* species using the baiting method described in Chapter 2. Two trials were conducted as an experimental repeat. A Chi-square test was used to look for significant differences between expected and observed counts for each separate variable: the two types of media, the presence or absence of a host, the pathogen species and the month of isolation (Minitab Statistical Software, release 15.1; Minitab Inc., State College, PA).

In general, *Phytophthora* spp. were able to persist in the soil for at least 12 months (Table A.2). Overall recovery rates at 1, 3, 6, 9 and 12 months were 96%, 98%, 98%, 90% and 76% respectively. No significant differences were found based on the pathogen species, the presence or absence of lilac, the type of medium or the month of recovery ($p=0.984$, $p=0.571$, $p=0.508$, $p=0.356$, respectively).

Phytophthora cactorum, *P. citricola* A and *P. citricola* B can persist under greenhouse conditions for at least 12 months regardless of host presence in both potting media and top soil. The fact that month 12 had a lower recovery rate (76%) may indicate that inoculum levels in the soil begin to taper off. However, our results did not show recovery at 12 months to differ significantly ($p=0.356$). These results are dramatically different than what was found by Linderman and Davis (4) who found 0% recovery of *P. cactorum* and *P. citricola* from garden clay loam soil after just one month. While the infestation and recovery methods were similar in this study, Linderman and Davis (4) did not include rice in their vermiculite/v8 spawn. Additional studies by Linderman and Davis (4) using infested rhododendron leaf pieces to infest soil found that both species could persist for up to 5 months, suggesting that the presence of plant material is an important factor in survival in the soil. Other studies have shown the persistence time to be much longer. Horner and Wilcox (3) found that *P. cactorum* could persist at least 18 months, and studies of other species have supported the hypothesis that *Phytophthora* spp. can persist for more than a year (8, 10).

Table A.2. Recovery rate of isolates from artificially infested media. Lilacs (*Syringa vulgaris*) were used as host plants. For each isolate-medium-host treatment in each month, n=8. Using the Chi square test, no significant differences were found by media types, host presence or absence, or pathogen species, or month of recovery.

Treatment			Recovery rate (%)				
Isolate	medium	host	Month 1	Month 3	Month 6	Month 9	Month 12
control	Cornell mix	yes	0	0	0	0	0
		no	0	0	0	0	0
	top soil	yes	0	0	0	0	0
		no	0	0	0	0	0
NYfs24 (*P. cactorum*)	Cornell mix	yes	100	100	100	100	87.5
		no	100	100	100	100	75
	top soil	yes	87.5	100	100	75	62.5
		no	100	100	100	100	87.5
NYfs11 (*P. citricola* A)	Cornell mix	yes	100	100	100	100	75
		no	100	100	100	100	100
	top soil	yes	100	100	87.5	100	0
		no	100	87.5	100	100	100
NYas2 (*P. citricola* B)	Cornell mix	yes	100	100	100	100	87.5
		no	100	100	100	75	87.5
	top soil	yes	100	100	100	87.5	75
		no	87.5	100	100	100	75

REFERENCES

1. Boodley J. W., and Sheldrake R. 1977. Cornell peat-lite mixes for commercial plant growing. Cornell University Cooperative Extension Division Information Bulletin 43.

2. Erwin D. C., and Ribeiro O. K. 1996. *Phytophthora* diseases worldwide. APS Press, St. Paul, Minn.

3. Horner I. J., and Wilcox W. F. 1996. Spatial distribution of *Phytophthora cactorum* in New York apple orchard soils. Phytopathology 86:1122-1132.

4. Linderman R. G., and Davis E. A. 2006. Survival of *Phytophthora ramorum* compared to other species of *Phytophthora* in potting media components, compost, and soil. Horttechnology 16:502-507.

5. Packer A., and Clay K. 2000. Soil pathogens and spatial patterns of seedling mortality in a temperate tree. Nature 404:278-281.

6. Shea S. R., and Broadbent P. 1983. Developments in cultural and biological control of *Phytophthora* diseases. Pages 335-350 in: Erwin D. C., Bartnicki-Garcia S., and Tsao P. H., eds. *Phytophthora*: Its Biology, Taxonomy, Ecology, and Pathology. APS Press, St. Paul, Minnesota.

7. Weiland J. E., Nelson A. H., and Hudler G. W. 2008. Aggressiveness of *Phytophthora cactorum* and *Phytophthora citricola* isolates on European beech and lilac. Phytopathology 98:S168-S169.

8. Weste G. 1983. Population dynamics and survival of *Phytophthora*. Pages 237-257 in: Erwin D. C., Bartnicki-Garcia S., and Tsao P. H., eds. *Phytophthora*: Its Biology, Taxonomy, Ecology, and Pathology. APS Press, St. Paul, Minnesota.

9. Wilcox W. F., and Mircetich S. M. 1984. Pathogenicity and relative virulence of seven *Phytophthora* spp. on mahalen and mazzard cherry. Phytopathology 75:221-226.

10. Zentmyer G. A., and Mircetich S. M. 1966. Saprophytism and persistence in soil by *Phytophthora cinnamomi*. Phytopathology 56:710.